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Relations between Hydrochloric Acid Secretion and Electrical Phenomena in Frog Gastric Mucosa

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For over a hundred years the existence of a potential difference (p.d.) across the stomach wall has been recognized, and there has been sporadic speculation as to the relation, if any, between this p.d. and the secretory activity of the gastric mucosa. The p.d. was first recorded by Donné (1834) who, discussing these 'courants électriques' asked 'sont-ils capables de rapprocher, de séparer les molécules l'une de l'autre, et de donner naissance à des corps nouveaux, comme cela arrive dans le règne inorganique?' This question still remains unanswered in spite of a large amount of work by numerous investigators. The early experiments, using primitive techniques and uncontrolled conditions, have been reviewed by Biedermann (1895). Later work, some with more refined techniques, but still using the stomach *in situ*, have been discussed by Rehm (1944) and by Rehm & Hokin (1947). In the present investigations isolated frog gastric mucosa was chosen as the experimental material because it can secrete hydrochloric acid *in vitro*, under conditions capable of controlled variations, and because it is possible to obtain information about its metabolic activity (Davies, Longmuir & Crane, 1947). These investigations into a possible correlation between electric properties and metabolism were initiated in 1945, after it had become clear that the hydrogen ions of the acid could not be produced directly by the fermentation or oxidative degradation of any carbon compound (Davies, 1948; Davies *et al.* 1947).

This paper deals with the techniques and apparatus used, and presents the results of experiments which show that there is a general correlation between acid secretion and the p.d. across the mucosa, the resistance of the latter and its ability to produce electric power externally. This is taken to indicate that the elaboration of hydrochloric acid is an electrochemical phenomenon. A hypothesis of acid production is presented which is in accordance with the known biochemical and biophysical observations. Accounts of investigations into the effects of direct electric currents on acid production and the effects of metabolic activators and inhibitors will be given in subsequent papers.

A part of the present work has been communicated to the Biochemical Society (Crane, Davies & Longmuir, 1946).

EXPERIMENTAL

Material. Frogs (*Rana temporaria temporaria* L.) were used in these experiments. They were captured locally, the web tattooed for identification, and were housed in a concrete froggery at 15–17°. An excess of cockroaches or worms was provided for food.

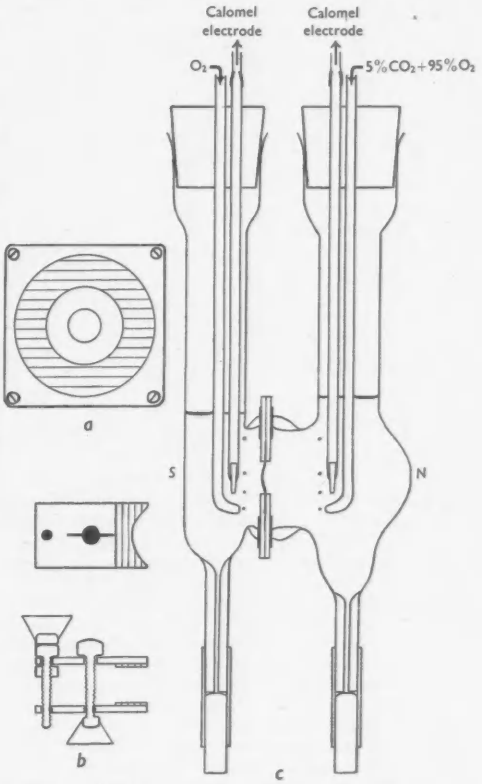


Fig. 1 *a*, Perspex holder for supporting mucosa; *b*, clamp for Type I and Type II vessels; *c*, Type I vessel for measuring p.d. across gastric mucosa and amounts of acid secreted. *N* = nutrient vessel; *S* = secretory vessel.

Preparation and mounting of tissue. The gastric mucosa was washed and isolated according to the method of Davies (1948) and the tube of mucosa then cut along the lesser curvature. Holders consisting of a pair of perspex sheets 1·5 mm. thick and 4 or 5 cm. square with a central hole were used for supporting the mucosa (see Fig. 1*a*). The holes, from 3 to 12 mm. diameter, were surrounded on the inner sides by concentric grooves in the perspex which gripped the tissue. Rubber gaskets fixed on the outer sides prevented leakage at the joints with the glass vessels described below. The mucosa was laid across the hole between the two sheets and firmly clamped in position by tightening the four corner screws. In order to insulate it electrically the edges and screws of the holder were dipped in melted dental wax.

Each saturated KCl bridge consisted of two 15 cm. lengths of 2 mm. bore glass tubing connected by 30 cm. of bicycle-valve rubber tubing. The glass tubes were constricted at each end, and the lower end contained a tapered glass stopper 1 cm. long ground into the tube. The narrow end of the stopper protruded a few millimetres beyond the tube (Fig. 1*c*). These bridges were designed because agar bridges, soaked strings and filter papers proved unsatisfactory. They were flexible and easily flushed, refilled and cleaned, and their leak was not more than 10^{-4} ml./hr. with an excess pressure of 1 cm. of saturated KCl solution. Their resistance was about 50,000 Ω.

The bridge from each vessel led to a test tube containing a reference electrode at 25·0°, and the p.d. between the two

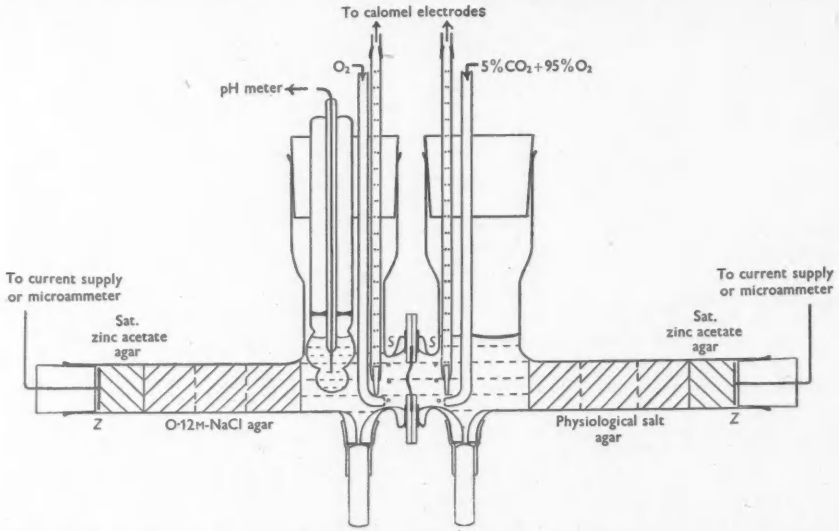


Fig. 2. Type II vessel for measuring pH of secretory solution, p.d. and resistance of mucosa, and for passing current through it. Oblique side-tubes for locating bridges attached at *SS*. *ZZ* = zinc-zinc acetate electrodes.

Vessels. The above unit was placed between the ground flanges of two glass vessels, which were then held together by a brass clamp with rubber-lined jaws (Fig. 1*b*). Several kinds of vessels were developed which were of two main types: Type I for measuring the p.d. across the mucosa and the amounts of acid secreted, and Type II for measuring additional quantities. Type I vessels are comparable with those of Delrue (1930) and of Gray, Adkison & Zelle (1940) and are shown in Fig. 1*c*. The nutrient vessel *N* contained about 36 ml. of nutrient solution (see p. 323) gassed with 5% CO_2 + 95% O_2 (v/v). The secretory vessel *S* contained about 14 ml. of 0·12M-NaCl solution gassed with O_2. There was an excess pressure of about 1 cm. of solution on the nutrient side. The capillary draining tube at the foot of each vessel was closed by a glass rod attached by rubber tubing. Each bung supported by means of small rubber collars a funnel, a glass bubbler and one end of a saturated KCl bridge. The bubbler was a narrow glass tube delivering a fine stream of either O_2 or 5% CO_2 + 95% O_2 previously wetted and warmed to 25·0°.

electrodes was measured by a potentiometer described later (see also Fig. 3). In early experiments Ag-AgCl electrodes (Brown, 1934) in 0·1M-KCl solution were used. Later, calomel electrodes were used in saturated KCl solution, the level of which was kept slightly above that in the vessels and storage test tube. The end *A* of each bridge was kept in this tube containing 0·12M-NaCl solution at 25·0°, except during measurements. With this precaution diffusion potentials were satisfactorily eliminated. Checks were frequently made that the p.d. between the two reference electrodes was zero with both bridges connected by the same solution, and showed calomel electrodes to be completely reliable and more satisfactory than Ag-AgCl electrodes.

Type II vessels (Fig. 2) were designed for the measurement of the pH of the secretory solution, the p.d. and resistance of the mucosa, and for passing current through it by means of the non-polarizable zinc-zinc acetate electrodes *ZZ*. These were made by rubbing saturated Zn amalgam on to disks of pure cleaned Zn. They had a constant resistance for current densities up to about 50 ma./cm.^{2}

Before each experiment the horizontal tubes were filled with four layers of 5% agar, the first made with saturated zinc acetate solution and the other three with physiological salt solution (see p. 323) or 0·12 M-NaCl solution. With this method no Zn was ever detected in the vessel solutions with 8-hydroxyquinoline or sodium diethyldithiocarbamate, and mucosae behaved normally. If all the saline agar was poured in together, so much zinc acetate dissolved in it that a toxic quantity subsequently reached the mucosa.

Bubblers were similar to those in Type I vessels. Bridges were similar, but for more accurate resistance measurements some of the Type II vessels were provided with side tubes inclined at 30° to the vertical (not shown in Fig. 2) which mounted in the bath was 20–30 min. This period was found to be well within the safety limit for normal subsequent behaviour.

Saline medium. The solution used during the washing and preparation of the mucosa was similar to the physiological salt solution of Krebs & Henseleit (1932) diluted by 0·29 vol. of water, except that it had a HCO_3^- content of 0·004 M and, under working conditions, a pH of 7·4. The nutrient solution was that of Krebs & Henseleit diluted by 0·29 vol. of water and containing 0·2% glucose. In aerobic experiments it was gassed with 5% CO_2 + 95% O_2, in anaerobic experiments with 5% CO_2 + 95% N_2. Analyses showed that our cylinders of this mixture contained 0·3–0·5% O_2.

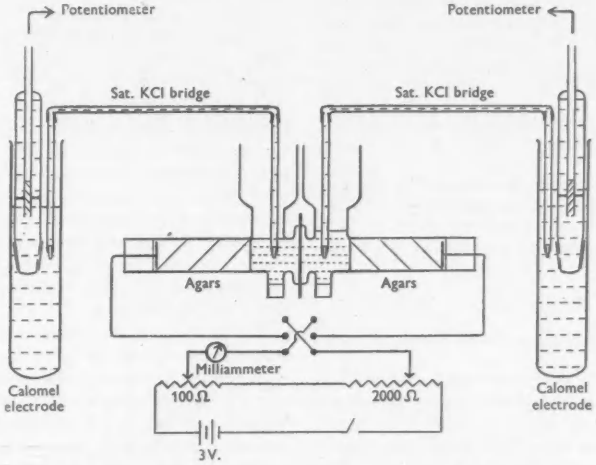


Fig. 3. Diagram of circuits for measuring p.d. and passing current through mucosa.

located the bridges accurately. This allowed precise determination of the correction due to the resistance of the solution. In experiments where a low correction was desirable, large Type II vessels were used, the horizontal tube having a diameter of 2·4 cm., nutrient and secretory volumes 25 ml. Where a low secretory volume was needed to give a higher acid concentration, vessels of 1·2 cm. diameter were used; the nutrient volume was 7 ml. and secretory volume 4–6 ml. The glass electrode (Marconi TM. 388 A) was mounted in the secretory vessel, on the far side of the bridge and bubbler from the mucosa. It was connected to a Marconi pH meter Type TF. 5511 C (see Fig. 2), and a saturated KCl bridge connected the secretory solution to a calomel reference electrode at 25·0°. The p.d. was measured as in Type I vessels.

Both types of vessel were immersed in a water bath at 25·0°, which also contained the reference electrodes, storage tube and gas manifolds. The temperature was kept uniform by water circulated through a warmed flask by air from a small motor blower; a second air stream stirred the water in the bath. In order to prevent the possibility of interference with electrical measurements electrical heating was avoided. The time elapsing between the pithing of the frog and the commencement of the experiment with the vessels

Measurement of p.d. and resistance. When measuring the p.d. across the mucosa it is desirable that virtually no current pass through it. The p.d. recorded is then the e.m.f. of the mucosa, and the mucosa is unaffected by the measurement. Two potentiometers, both null instruments, were used: in early experiments a Cambridge Electrometer Valve Potentiometer 44236, and later a battery-operated Marconi pH meter and potentiometer Type TF 511 C. The negative (secretory) side of the mucosa was connected to the grid of a valve and the positive (nutrient) side to the cathode circuit. The valve in each of these instruments has a very high grid-cathode resistance, at least 10^9 Ω, so that the current through the mucosa was entirely negligible. The p.d. was determined to ±1 mV. except in very early experiments when the accuracy was ±2 mV. The resistance of the mucosa was determined by measuring the p.d. between the two saturated KCl bridges when a small current was passed through the membrane by means of the two zinc-zinc acetate electrodes. By measuring the p.d. between points near the mucosa the resistance correction was kept small, and by passing the current between electrodes covering the whole cross section of the tubes, uniform current flow was obtained. A potential divider provided currents from 0·01 to 10 ma. (see Fig. 3), which were measured by multiple range meters

with ranges from 0·1 ma. upwards. Calibration showed them to be accurate to 1 % (2 % for lowest range). The value of the correction for the resistance of the solutions was calculated and also determined experimentally for each pair of vessels.

Estimation of acid secreted. In early experiments the secretory solution was periodically removed and its acid content determined manometrically. In later experiments when a pH meter was available, the pH of the secretory solution was also measured *in situ* as described above. In the first method, a 6 ml. sample of the secretory solution consisting of 0·12M-NaCl together with any secretion, also 0·12M, was placed in the main compartment of a Warburg cup, and 0·5 ml. of a solution containing 0·02M-$NaHCO_3$ and 0·10M-NaCl was placed in the side arm. After equilibration with 5 % CO_2 + 95 % O_2 at 25° the solutions were mixed and the output of CO_2 was observed. This determination, to about pH 6, was compared with those from control solutions of NaCl + HCl of total strength 0·12M, but of varying acidity. This procedure is necessary because it has been found that CO_2 is less soluble in acid than in neutral solutions of equivalent concentrations (Davies & Longmuir, 1947). The 'isotonic' $NaHCO_3$ was used to keep the blanks as low as possible.

The dry weight of the mucosa was obtained by removing the tissue quantitatively from the hole in the perspex holder, washing in distilled water and drying overnight at 110°. The Q_{HCl} was then calculated, and is given in μl./mg. dry wt./hr.

RESULTS

Electrical properties of non-secreting gastric mucosa

Potential difference. There was a p.d. across all of 150 frog gastric mucosae, the secretory side being in every case negative (in an external circuit) with respect to the nutrient side. Each experiment lasted between 6 and 12 hr. after the vessels containing the mucosa were mounted in the water bath, this instant being taken as zero time. During the first 0·5 hr. the p.d. between the secretory and nutrient sides usually increased in magnitude (Figs. 6–8). We have found that after slight mechanical injury to either side of the mucosa the p.d. was temporarily reduced in magnitude by 2–15 mV. (eight experiments). The initial rise is thus probably due to the decrease of injury potentials resulting from dissection and mounting, and we are not primarily concerned with it in these experiments. It occurred in seventy-two out of eighty-two experiments; the average initial p.d. of the secretory side was −20 mV. and the average maximum value −32 mV. After this peak the p.d. dropped slightly and remained rather steady for many hours in an untreated mucosa unless acid secretion occurred.

In ninety-four experiments the steady p.d. varied between −7 and −50 mV., with a mean value of −30 mV. and a standard deviation of 9 mV. There was no significant difference between males (69) and females (25), nor between fasting frogs and those actively digesting at the time of pithing. Nor was any correlation apparent with the weight of the frog. There was, however, a difference between summer and winter frogs, in spite of the fact that the latter were kept warm and accepted food. From March to June 1946 the mean p.d. across thirty-seven mucosae was −35 mV. and the standard deviation 7 mV.; from September to February the mean p.d. across fifty-seven mucosae was −27 mV. and the standard deviation 9 mV. Mucosae of frogs which had been in captivity over a month usually gave rather a low p.d., and were in general less active than those of freshly caught frogs. To overcome this difficulty frogs are now kept out of doors under natural conditions.

Resistance. The resistance of 1 cm.² of thirty-nine mucosae varied between 90 and 310 Ω-cm.²; the mean value was 210 Ω-cm.² and the standard deviation 60 Ω-cm.² Taking the mean thickness as 0·25 mm., these results give for the mean specific resistance of frog gastric mucosa 8×10^3 Ω-cm. and the standard deviation 3×10^3 Ω-cm. It should be noted, however, that in all cases the area used was that of the hole in the perspex holder. Since the mucosa is essentially non-planar, these areas are minimum values, and represent between 0·5 and 1·0 times the true area. For this reason the unit of mucosa used in power calculations was not 1 cm.² but 1 mg. dry weight.

For both p.d. and resistance the standard deviation was about 30 % of the mean value. No significant variation of the resistance for unit area or for 1 mg. dry weight was observed with any of the variables mentioned in the last paragraph but one, nor with the area of mucosa used. Mucosae with a low specific resistance, however, never attained such high rates of acid secretion as some with high specific resistance (fifteen mucosae), and the magnitude of the specific resistance seemed to give rather a good indication of the 'state of health' of a mucosa at any particular time. The initial rise in p.d. was accompanied by a rise in resistance representing about 20 % of its steady value (fifteen experiments). Injury to the mucosa reduced both p.d. and resistance. Death was accompanied by a rapid decrease of the p.d. to zero, and of the resistance to a small fraction of the live value. For mucosae killed by hydrochloric acid or hydrogen cyanide the dead resistance was 10–30 % of the live resistance; for mucosae killed by heat (30 min. at 60°) not more than 5 %. An interesting observation is the effect of iodoacetate. During 1–2 hr. after its addition to 0·0015M the p.d. was abolished and the resistance rose to about twice its normal value. Subsequently, the p.d. remained zero and the resistance dropped in 0·5 hr. to the dead value.

Electrical power output. An important observation is the constancy of the resistance for currents below about 1 ma./cm.² This was true for applied currents both enhancing and opposing the natural p.d.,

and also for currents maintained by the e.m.f. of the mucosa itself when no other source of power was included in the circuit. Results for one mucosa are shown in Fig. 4. As in all other similar experiments, the points obtained with an applied current, and those obtained when the mucosa itself provided the power, lie on the same straight line within experimental error; the mucosa obeyed Ohm's Law. Moreover, when the two sides were connected electrically (through the solution, agar, electrode and galvanometer resistances) a current was maintained without appreciable polarization. The greatest current observed when the mucosa was the only source of electrical power in the circuit was 0·07 ma. This occurred when the resistance in the circuit apart from the mucosa (120 Ω) was 460 Ω. The electrical power output of the mucosa was then $(0{\cdot}07)^2 \times 580$ μW., or 2·8 μW. The effective area of the mucosa was 1·8 cm.² allowing for bellying, and its dry weight 10·8 mg. The greatest observed power output was thus 1·6 μW./cm.² or 0·26 μW./mg. dry weight. The magnitude of the external resistance precluded the observation of a higher power output.

However, when sheets of mucosa were immersed in a beaker of nutrient saline solution at 25·0° the p.d. across them, measured between two points close to them, was maintained without polarization at about one fifth of the open circuit value, so that the power developed was 11 μW. This suggests that if the resistance external to the mucosa could be reduced to zero, the total electrical power, (p.d. across mucosa)²/resistance of mucosa, would be developed. In the case considered above this maximum power would be $40^2/120$ μW., i.e. 13 μW. This is also equal to (intercept on current axis)² × resistance of mucosa = $(0{\cdot}33^2 \times 120)$ μW. The maximum power is thus 7 μW./cm.² or 1·2 μW./mg. dry weight.

Electrical properties of some other parts of the gastro-intestinal tract

Various neighbouring parts of the digestive tract of the frog were examined. Across oesophageal, pyloric, duodenal and ileal mucosa (fourteen experiments) no p.d. was found comparable in magnitude with that across mucosa from the corpus of the stomach. The highest p.d. observed was that across the pyloric mucosa, which in eight experiments gave an average of −5 mV. The other membranes had a mean p.d. of −2 mV. (six experiments). In all cases, as in the corpus, the secretory side was negative with respect to the nutrient side. The resistance of oesophageal, pyloric, duodenal, ileal and rectal mucosa was determined in six experiments only: all values were within the range found for the corpus. This is also true of their specific resistance and resistance for 1 mg. dry weight.

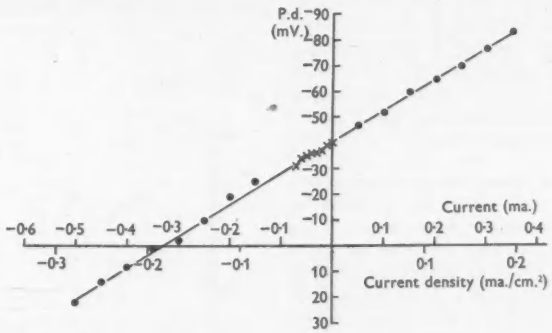


Fig. 4. Relation between p.d. across a frog gastric mucosa and current through it. ×—× with mucosa as only source of power; •—• with external source of power.

Experiments with three rectal mucosae gave steady p.d. values of −8, −12 and −27 mV. The muscle layer dissected off from frog gastric mucosa was also investigated: the p.d. across it was zero in all of five experiments. The resistance of unit area was measured in three cases, giving a mean of 70 Ω-cm.² Since the thickness was about 0·5 mm. the specific resistance was 1400 Ω-cm., considerably lower than that of the gastric mucosa.

Experiments in the absence of oxygen

Experiments on nine mucosae have shown that an adequate supply of oxygen is necessary to maintain both acid secretion and the p.d. across the membrane. Their mean p.d. with an oxygen pressure of 1 atm. was −27 mV.; this was reduced to −3 mV. when the oxygen was replaced by nitrogen (containing from 0·3 to 0·5 % oxygen), and in four cases the p.d. fell to zero. The abolition of the p.d. was

reversible, and recovery occurred in *all* cases on supplying oxygen, even after several hours of anaerobiosis. The whole cycle could then be repeated using the same mucosa. When the experiment was begun under anaerobic conditions, there was no initial rise in p.d., which instead fell rapidly to a very low value as the oxygen in the tissue became depleted. Fig. 5 gives a typical curve for two complete cycles.

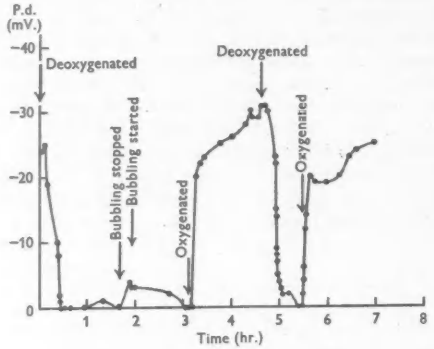


Fig. 5. Effect of anaerobiosis on p.d. across a frog gastric mucosa.

The resistance of the tissue was increased in the absence of oxygen in both of two experiments by about 50%. It fell again to approximately its previous value on subsequent oxygenation. The secretion of acid by the mucosa was inhibited concomitantly with the decrease in p.d., and in no case was any hydrochloric acid produced anaerobically.

Secretion of acid by frog gastric mucosa

In dealing with the secretion of acid by frog gastric mucosa it was found that the seasonal variations in the behaviour of frogs both presented problems and gave a wide range of material from which to choose. In late spring and summer, about half the mucosae secreted acid spontaneously, and after the addition of histamine (5×10^{-5} M) to the nutrient solution most others secreted acid. Histamine also often increased the rate of acid secretion of spontaneously secreting mucosae. The Q_{HCl} had values up to 8·0. In winter, fewer mucosae secreted acid and none did so spontaneously. The average rate was lower than in summer, and the maximum Q_{HCl} observed was 4·6. There was a period in spring, however, when summer conditions were not yet fully established, and almost no mucosa secreted spontaneously, but all did so after the addition of histamine, whenever this was added (usually between 1 and 7 hr. experimental time, Figs. 6–8). When spontaneous secretion occurred, it most often started at about 2 hr. experimental time. In some winter and spring mucosae secretion stopped on the removal of histamine, and secretion could subsequently be reinitiated by the addition of more histamine.

We wish to introduce the term 'secretive' for a mucosa capable of secreting acid, either spontaneously or during stimulation, whether this mucosa is actually secreting or not. The term 'non-secretive' is used for a resistant mucosa, i.e. one which cannot be made to secrete acid even by the addition of histamine.

No acid secretion was detected in oesophageal, pyloric, duodenal, ileal or rectal mucosa (eight experiments).

Relation between potential difference, resistance and acid secretion

The commencement of acid secretion, whether spontaneous or induced by histamine, was accompanied by a drop in the p.d. across the gastric mucosa. This drop also accompanied a histamine-induced increase in the rate of secretion of a mucosa

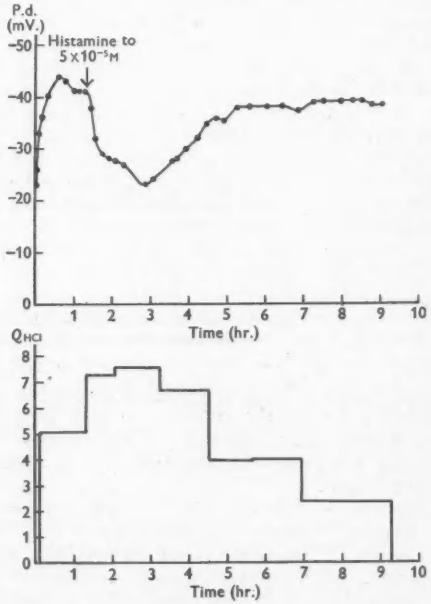


Fig. 6. P.d. and Q_{HCl} of a frog gastric mucosa. Spontaneous secretion and effect of histamine.

already secreting spontaneously. (In Fig. 6 spontaneous secretion occurred during the initial rise in p.d., which masked the effect.) This decrease in magnitude of the p.d. was usually higher for high rates of secretion than for low ones, being of the order of 10 mV. for a Q_{HCl} increase of 2, or 17 mV. for

a Q_{HCl} increase of 6. The effect was reversed when secretion stopped, either spontaneously (Fig. 6) or on removal of histamine (Fig. 8), or by the addition of thiocyanate (0·012 M) to the nutrient solution, which inhibited acid secretion (Fig. 7).

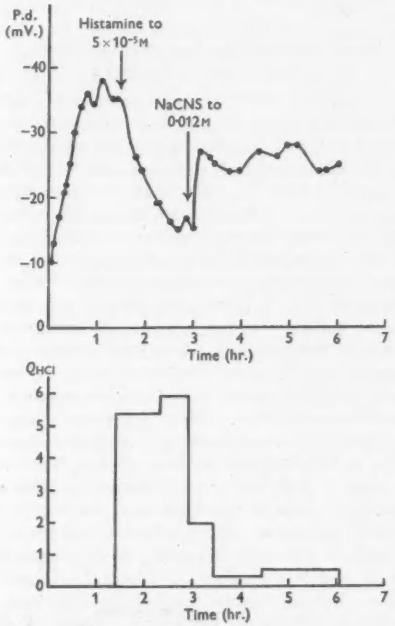


Fig. 7. Effect of histamine and thiocyanate on p.d. and Q_{HCl} of a frog gastric mucosa.

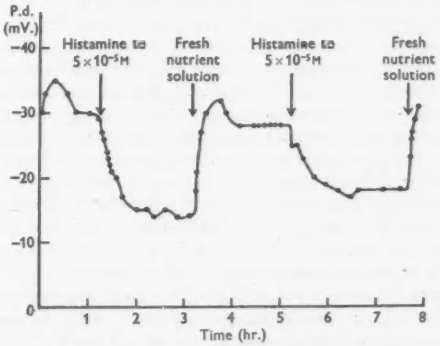


Fig. 8. Effect of addition and removal of histamine on p.d. of a (winter) frog gastric mucosa.

In summer, acid secretion sometimes commenced spontaneously before experimental time 0·5 hr., and in some cases it continued for periods up to 11 hr.

Fig. 6 gives results for a spontaneously secreting mucosa in April, which also responded to histamine and secreted for 9 hr., the rate falling off after the third hour. The maximum Q_{HCl} (over 1 hr.) was 7·6 and the total amount of acid secreted 435 μl. Fig. 7 shows results for a secretive mucosa in March, which did not secrete acid until histamine was added at experimental time 1·5 hr. In this mucosa, acid secretion was almost completely inhibited after 1·5 hr. by the addition of 0·012 M-thiocyanate which does not inhibit respiration (Davies & Terner, 1948), and the p.d. rose at the same time. Fig. 8 refers to a mucosa in December which responded twice to histamine, the effect being reversed both times when the histamine was removed. In this experiment nutrient saline was used on both sides of the mucosa, and the acid was not estimated.

The variation of the resistance of the mucosa when acid secretion commenced (or was stimulated by histamine) was followed in six experiments, four of which were concerned with the actual onset of secretion. In all cases the resistance rose while the p.d. fell, and the fractional rise in resistance (x) increased both with the fractional fall in p.d. (y) and with the actual fall. In three cases x and y were both below 15%, in two cases both were between 30 and 40% and in the remaining case $x = 20\%$ and $y = 11\%$. x and y were thus of the same order of magnitude in each case.

Diffusion potentials

The possibility was investigated that the change in p.d. across the mucosa at the onset of acid secretion is partly a diffusion potential due to acid in the tubules of the mucosa. Two types of experiment were carried out. (*a*) The 0·12 M-sodium chloride on the secretory side of a non-secreting mucosa was replaced by 0·12 M-hydrochloric acid, and after more than 10 min. this was replaced by 0·12 M-sodium chloride, the p.d. changes being watched carefully during the second change-over. The 0·12 M-hydrochloric acid left in the gastric crypts by this treatment is likely to take some minutes to diffuse completely into the new solution, and a p.d. level during this interval comparable with the p.d. after the onset of secretion would support the hypothesis stated above. No such temporary drop was observed. (*b*) A model was set up,

Hg.Hg_2Cl_2 | sat. KCl | 0·12 M-NaCl | cellophan | sintered glass disk | 0·12 M-NaCl | sat. KCl | Hg_2Cl_2.Hg,

to represent the system

Hg.Hg_2Cl_2 | sat. KCl | nutrient solution | muscularis mucosae | glandularis mucosae | secretory solution | sat. KCl | Hg_2Cl_2.Hg.

When the 'secretory' sodium chloride was replaced by 0·12 M-hydrochloric acid the p.d. changed immediately from 0 to -20 mV. and rose to -32 mV.

during the next 5 min. while the acid diffused through the sintered glass to the cellophan. After 1·5 hr. the p.d. had fallen to −29 mV. When the hydrochloric acid was replaced by sodium chloride, the p.d. fell within 5 sec. to −2 mV., and never changed sign, as would have been expected if the acid in the 'tubules' of the sintered glass caused asymmetrical diffusion potentials. In both these experiments care was taken to avoid momentary diffusion potentials at the bridges by rinsing the end in saturated potassium chloride solution before each p.d. measurement in a new solution.

It was important to know whether, at the pH values of the secretory solution obtained with secreting mucosae, diffusion potentials were produced which affected the measured p.d. In six experiments hydrochloric acid solutions of pH 4, 3 and 2 were used consecutively on the secretory side of a non-secreting mucosa in place of 0·12M-sodium chloride. In no case did a solution of pH 4 or 3 produce any change in p.d. greater than 2 mV., whether or not it was made isotonic by the addition of sodium chloride. Hydrochloric acid (0·01M) produced no greater effect on mucosae when added within several hours of mounting, but in some cases increased the magnitude of the p.d. by up to 20 mV. when added after 6 hr. experimental time. These hydrochloric acid solutions had no effect on the p.d. of a secreting mucosa.

In our experiments the pH of the secretory solution never fell below 3; moreover, when the secretory solution of a secreting mucosa was replaced by fresh sodium chloride solution, there was no observable change in the measured p.d. The secretory solution was always replaced before its pH fell below 3, and in view of all the above evidence it seems impossible to account for the p.d. changes during secretion on the basis of simple diffusion potentials.

DISCUSSION

Sign of potential difference across gastric mucosa

Previous workers, including Rosenthal (1865), Bohlen (1894), Biedermann (1895), Mond (1927), Rehm (1943, 1944) and Teorell & Wersäll (1945), found with the stomachs of a variety of animals that in an external circuit the secretory surface was at a negative potential with respect to the serosa, and that the p.d. diminished with the onset of acid secretion. Delrue (1935) claimed opposite signs for the p.d. and its change on secretion; it is probable that he was considering the direction of internal rather than external currents.

Injury potentials

The rapid increase in the magnitude of the p.d. across the mucosa after mounting is probably caused by the decrease of injury potentials which are in the opposite direction to the natural p.d. The accompanying increase in resistance is not great enough to account, by the reduction of local currents through the damaged edges as the tissue becomes stabilized, for more than a small fraction of the p.d. rise. Injury potentials of dog stomach have been discussed by Rehm (1944).

Relationship between potential difference, resistance and acid secretion

When acid secretion commenced, either spontaneously or after histamine, the magnitude of the p.d. fell by an amount greater for high than for low rates of acid secretion. The experiments on diffusion potentials support the view that not more than a small part, if any, of this fall can be due to a diffusion potential caused by the presence of hydrochloric acid either in the secretory solution or in the gastric tubules. It is clear from the data of Rehm (1944) and Rehm & Hokin (1947) that the pH in the secretory chamber attached to an actively secreting dog's stomach could fall from above 5 to 2 without any change in the p.d. across the stomach wall. Teorell & Wersäll (1945) recorded only small changes in the p.d. across secreting isolated frog gastric mucosa when the pH of the secretory solution was changed from 4 to 2 by the addition of hydrochloric acid. They also noted that the secretory surface was 'insensitive towards the ionic composition of the solutions bathing it'. We also have found that wide variations in the ionic composition of the secretory solution had little effect on the p.d. across the mucosa. Similar results have been obtained by Rice & Ross (1947), who have reviewed the literature on this subject.

The rise in resistance which accompanies the p.d. drop at the onset of acid secretion, and seems to be quantitatively related to it, suggests that the electrical structure of gastric mucosa is changed when acid secretion commences, and that the p.d. fall is due to more fundamental changes than superimposed diffusion potentials. While many experimental procedures have been found which change both the resistance of live frog gastric mucosa and the p.d. across it, none was found which affected only one of these. Teorell & Wersäll (1945) recorded a rise in both p.d. and resistance of isolated frog gastric mucosa between the time of mounting and the commencement of acid secretion about 1 hr. later. They did not, however, differentiate between the injury effects due to mounting and changes accompanying the onset of secretion.

When acid secretion finished, either spontaneously or as a result of the addition of thiocyanate (0·012M) the magnitude of the p.d. rose again towards its previous value. Davies & Terner (1948) found that at this concentration thiocyanate had no effect on the rate of oxygen uptake of isolated frog gastric

mucosa, and that it neither prevented nor abolished the increased rate of oxygen uptake due to 5×10^{-5} M-histamine. In one experiment, when histamine was added to the nutrient solution of a secretive mucosa which already contained 0·01 M-thiocyanate, the magnitude of the p.d. increased by 6 mV.

On the other hand, when both the cyanide-sensitive respiration and acid secretion were abolished by 0·001 M-cyanide, the p.d. fell to zero and the resistance fell to 10–30 % of the original value. During anaerobiosis both respiration and secretion were also absent, but, whereas the p.d. fell rapidly to zero, the resistance increased by about 50 %. On subsequent oxygenation the p.d. and resistance returned to their previous values even after several hours of anaerobiosis. Both Rehm & Hokin (1947) and Rice & Ross (1947) found that the p.d. across a dog's stomach wall was dependent upon an adequate oxygen supply to the tissue. No workers have recorded acid secretion in the absence of respiration or a p.d. across the mucosa.

Our results further show that the dead/live resistance ratio for isolated frog gastric mucosa may be considerably greater than for other active biological material for which information is available. Cole (1947) gives the value for the dead/live membrane resistance of cells as a fraction of 1 %. Blinks (1930) found that the dead/live resistance for *Valonia ventricosa* cells was 1 or 2 %. The highly active oxyntic cells in gastric mucosa may, however, have a different dead/live resistance ratio from the whole tissue.

Potential difference and acid secretion of other regions of the gastro-intestinal tract

The p.d. across the oesophageal, pyloric, duodenal and ileal walls, and across the muscle layers dissected off the corpus of the stomach, was found to be small or zero and unaffected by histamine. It is of interest that of these regions only the acid-secreting mucosa had a high p.d., and that the p.d. was affected by histamine only if it was secretive.

Experiments on rectal mucosa are being extended, and will be discussed in a later paper. It should be borne in mind in connexion with the appreciable p.d. of this tissue (mean of three experiments -16 mV.), that frog gastric mucosa, frog skin (which shows an even higher p.d.) and frog rectal mucosa are all tissues concerned actively with water transport. The last two membranes transport an approximately neutral solution in the same direction as their external positive p.d., whilst gastric mucosa secretes an acid solution in the opposite direction to its external positive p.d.

In frogs, pepsin is largely or entirely secreted by the oesophagus (see Friedman, 1937). In Friedman's (1934) experiments with frogs the brain was destroyed and 'one third of the way up from the pylorus the stomach was divided to form a pyloric portion and a cardiac portion'. He found that when either the cardiac or pyloric part was stimulated with glass beads, acid was formed only in the pyloric region. No figures were published to support this claim. Using the technique described above, we have shown beyond any doubt that the corpus of the stomach produces acid, and have also failed to detect any acid secreted by the pyloric mucosa of a stomach whose corpus secreted acid. Friedman (1934) used *Rana esculenta*, and we have confirmed that in this species too acid is secreted by the corpus region of the stomach.

Electric power produced by isolated frog gastric mucosa

Donné (1834) first raised the question as to whether the electric currents observed in the stomach could do useful chemical and mechanical work. Brücke (1859) suggested that the electromotive forces in gastric mucosa may enable it to send an acid fluid towards the secretory surface and a basic fluid in the opposite direction. In 1872 Engelmann published experiments which are of interest in connexion with these suggestions. He studied the secretion of the glands in frog skin by drying the skin and observing microscopically when it became wet again. Although he did no quantitative work he showed that the glands secreted only when an e.m.f. could be detected, and that various agents which abolished the e.m.f. also abolished secretion. He suggested that the glands in the skin produced their secretion by electroendosmosis (Engelmann, 1872). Since then there has been much speculation, but until recently very little experimental evidence has been published, and this is more suggestive than conclusive. Some of the work has been reviewed by Keller & Pisha (1947).

Recently evidence has accumulated which supports the hypothesis that electrical energy is utilized in the production of acid by the stomach. It has been found that the rate of acid secretion of gastric mucosa can be increased by passing through it an electric current which enhances the natural p.d., and decreased by a current in the opposite direction (Rehm, 1945 (dogs); Crane *et al.* 1946; Crane & Davies, 1947*a* (frogs)). It has also been found that gastric mucosa can maintain an electrical power output without appreciable polarization (Rehm, 1943; Crane *et al.* 1946). The greatest observed power output of the frog gastric mucosa considered above (p. 325 and Fig. 4) was 0·25 μW./mg. dry weight, and the power output expected with zero external resistance was 1·2 μW./mg. dry weight. Davies & Longmuir (1947) found the average Q_{O_2} of fifty non-secreting isolated frog gastric mucosa to be 2·1. If we take as a first approximation the usual value of 0·005 cal./μl. oxygen for the oxidation of glucose (Brody, 1945), the average rate at which energy is

liberated in non-secreting frog gastric mucosa by glucose oxidation is $0{\cdot}0050 \times 2{\cdot}1$ cal./hr./mg. dry weight, or 12 μW./mg. dry weight (1 cal./hr. $= 1{\cdot}16 \times 10^3$ μW.). Using this value for the mucosa discussed above, the greatest electrical-power output observed represents 0·25/12, i.e. 2 % of the metabolic power, which with zero external resistance would be increased to 1·2/12, i.e. 10 %.

mately 10 %) in maintaining an electric current. This refers to a non-secreting mucosa and, since the p.d. is lower and the resistance higher during acid secretion, less power is then available to drive a current round an external circuit. This may be because electric power is being used internally to produce acid. The maximum electric power output of the mucosa considered earlier (p. 325), which was

Table 1. *Production of maintained electric power by various living tissues*

Author	Material	Temperature (° C.)	Approximate p.d. across membrane (mV.)	Greatest observed current density in active membrane (ma./cm.²)	Greatest observed electrical power output of active membrane (μW./cm.²)	Greatest observed electrical power output of active membrane (μW./mg. dry wt.)	Electrical power / Metabolic power (Author's figure) (%)
Blinks (1933)	Halicystis cell	25–30	70	0·01 ma./cell	0·7 μW./cell	—	—
Francis (1933)	Isolated frog skin	20	—	0·015	2·5	0·5	5–10
Stapp (1941)	Isolated frog skin	25	40	0·02	0·8	0·15	1–2·5
Rehm (1943)	Dog stomach	39	60	0·15	8·9	0·34	2–4·5
Crane *et al.* (this paper)	Isolated frog gastric mucosa	25	40	0·04	1·6	0·26	2

At least five workers besides ourselves have determined the electrical-power output maintained by living tissue, and some of the results are collected in Table 1. (The specialized tissue of electric organs of some fishes is excluded from the present discussion; the current varies rapidly and widely during a train of discharges.) All the results in Table 1 refer to experiments in which the two sides of the membrane were connected electrically through a circuit of fairly low resistance, and the current densities cited were maintained without appreciable polarization. Throughout, the power produced was limited by the external resistance, which differed in each case, and a higher power would be expected with zero external resistance. The last column gives an approximate value of the ratio: greatest observed electrical power output/estimated rate at which metabolic energy is liberated (the efficiency of the process). Whilst some of the figures in this table are based on incomplete data, it is of interest that the efficiencies are of the same order of magnitude. Lund & Stapp (1947) have recently published a series of results for frog skin, which give efficiencies (electrical power/metabolic power) of 1–2 %.

From a comparison of the structure, the p.d. and resistance of nearby regions of the gastro-intestinal tract, and from the relations previously described between the electrical properties of gastric mucosa and acid secretion, it seems likely that most, if not all, of the p.d. across this tissue is due to the oxyntic cells, but that their resistance is only a small part of the whole.

It is remarkable that a gastric mucosa can expend such a large fraction of its metabolic energy (approxi-

not secreting acid, could support a Q_{HCl} of the order of 2, i.e. about one quarter of the maximum Q_{HCl} observed in frog gastric mucosa. The relationship between the electrical energy and the energy used in secretion will be discussed elsewhere. It should be noted that more metabolic energy is available to the oxyntic cells during acid secretion because the rate of respiration is increased (Davies, 1948).

A SUGGESTED ELECTRON-CYCLE MECHANISM OF HYDROCHLORIC ACID PRODUCTION

Golgi (1893) found that the intracellular canaliculi form a network just below the surface of the oxyntic cells (shown schematically in Fig. 9*b*). Bradford & Davies (1948), using indicators, demonstrated that these canaliculi contained acid during acid secretion, thus showing that the acid is formed in the region between the canaliculi and the external cell wall (pericanalicular zone).

Chemical processes involved

The fundamental overall reaction concerned in hydrochloric acid production (Davies, 1948) is

$$H_2O \rightarrow H^+ + OH^-, \qquad (1)$$

and evidence has been presented in this paper and previously (Rehm, 1943, 1945; Crane *et al.* 1946; Davies *et al.* 1947; Crane & Davies, 1947*a*) which supports the theory that some of the energy available from metabolism within the oxyntic cells is utilized in an electrochemical process which results in the net separation of the electrically charged H^+ and OH^- ions.

We suggest that the cyclic mechanism described in the following paragraph, and shown in Fig. 10, is operative. The overall reaction is that which occurs when the well-known cell

hydrogen | OH^- aq. ⋮ H^+ aq. | hydrogen

(see MacDougall, 1939) is reversed by a source of power (Davies *et al.* 1947).

According to our hypothesis two coupled systems of enzymes are involved (*a*) to transport H atoms (electrons + protons) across the pericanalicular zone, and (*b*) to return the electrons to the starting point near the cell wall. Energy derived from aerobic metabolism drives a system composed of a series of dehydrogenating enzymes (*a*) whose prosthetic groups (such as coenzymes I and II) send H atoms

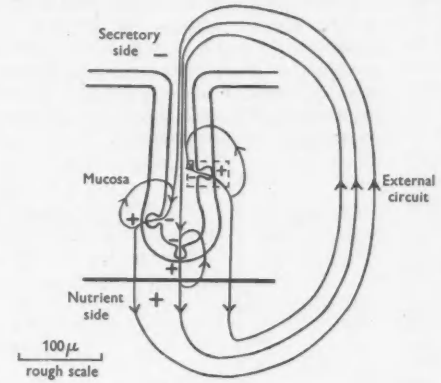


Fig. 9*a*.

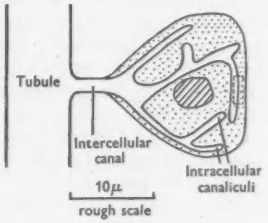


Fig. 9*b*.

Fig. 9. *a*, Diagram of gastric tubule showing lines of current flow across and in the mucosa due to its own e.m.f. Three oxyntic cells shown. *b*, Diagram of single oxyntic cell enlarged from *a* showing network of canaliculi. Sample cross-section of pericanalicular zone enclosed in rectangle.

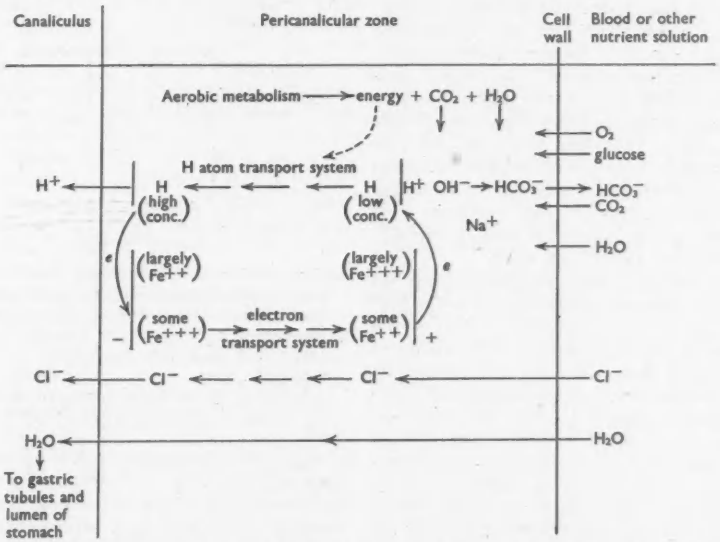


Fig. 10. Suggested electron-cycle mechanism of HCl production in the pericanalicular zone of oxyntic cells (enlarged from Fig. 9*b*). This schematic diagram is not drawn to scale; the H atom and electron transport systems probably occupy only a small fraction of the space between the canalicular and cell walls.

through the pericanalicular zone, thus maintaining a very low H atom concentration at the side nearest the cell wall and a very high H atom concentration at the canalicular wall. The electrons are returned by a coupled cytochrome or cytochrome-like system (*b*) (e.g. Fe^{++}-Fe^{+++}) oriented across the zone. A unit cycle can now be described. A H^+ ion formed from water near the cell wall oxidizes a Fe^{++} to a Fe^{+++} ion, and by accepting the electron becomes a H atom. The atom is transported to the region of high H concentration at the canalicular wall, this endergonic process being coupled with energy-giving reactions. The H atom then reduces a Fe^{+++} to a Fe^{++} ion; the resultant H^+ ion passes into the canaliculus together with the Cl^- ion released by reduction of the Fe^{+++} ion. The electron from the H atom is returned to the region of low H concentration near the cell wall by the potential of the Fe^{++}-Fe^{+++} system, and passes round the cycle again. Valency requirements are satisfied by the passage of a Cl^- ion across the system in the opposite direction to the electron (cf. Lundegårdh, 1945). The overall result for each electron cycle is thus reaction (1) with the transport of one Cl^- ion.

Previous work has shown that the residual OH^- ions from the water react (via the cytoplasmic buffers) and are converted by carbon dioxide from internal and external sources into HCO_3^- ions (Davies, 1948; Davies & Longmuir, 1948), this process being catalyzed by carbonic anhydrase (Davies & Roughton, 1948). It is clear, therefore, that this cyclic mechanism involves no *net* transfer of charge through the acid-secreting oxyntic cells or across the mucosa: at the cell wall Cl^- ions exchange with HCO_3^- ions, and at the canalicular wall H^+ and Cl^- ions are secreted (cf. Davenport, 1946).

Effect of histamine. The action of histamine could be explained by the assumption that it takes part as a link in the H atom transport system of the electron cycle by the tautomerism or resonance of its iminazole ring. A similar role for histidine has been suggested as part of the mechanism of cytochrome *c* activity (Theorell, 1947). The work of Hallenbeck, Code & Gregory (1947) supports the view that agents stimulating hydrochloric acid secretion act finally through histamine (cf. Babkin, 1944).

Crane & Davies (1947*b*) have calculated that in order to form 1 mol. of hydrochloric acid under the conditions obtaining in frog oxyntic cells, the system must be supplied with about $1{\cdot}0 \times 10^4$ cal. of free energy (a few hundred calories more are required in mammalian oxyntic cells). Lipmann (1941) and Meyerhof (1944) have calculated the free energy released by the hydrolysis of high-energy phosphate bonds. For ATP they found c. 10,000 and 12,000 cal./mol., respectively. It is interesting that the splitting of one such bond might thus be able to energize one unit electron cycle and thus produce one H^+ ion.

Electrical processes involved

Potential differences in oxyntic cells. The postulated electric circuits within the mucosa are shown schematically in Fig. 11*a* and *b*, which represent a sample cross-section of the mucosa containing a single secretory unit in the pericanalicular zone of an oxyntic cell. *E* is the e.m.f. which maintains the p.d. measured in our experiments.

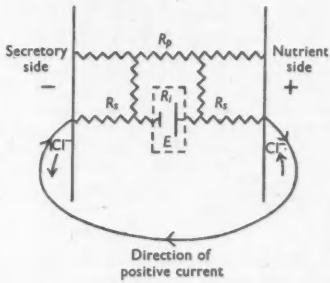


Fig. 11*a*.

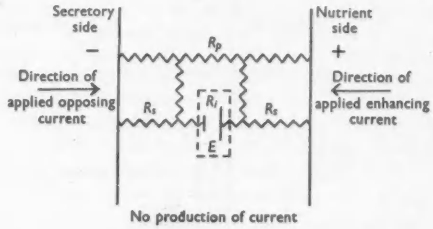


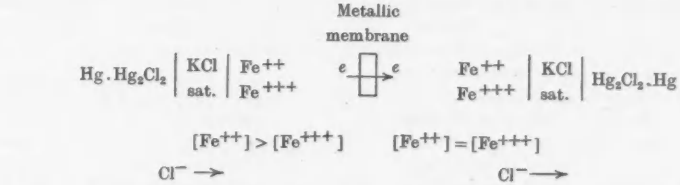
Fig. 11*b*.

Fig. 11. Suggested electric circuit diagram within sample cross-section of gastric mucosa. Enclosed portion represents the pericanalicular zone of the single oxyntic cell considered. *a*, Non-secreting mucosa (showing external current). *b*, Acid-secreting mucosa. E=e.m.f. of pericanalicular zone. R_i=internal resistance of pericanalicular zone. R_s=mucosal resistance in series with R_i. R_p=resistance of rest of sample cross-section of mucosa.

Since the pH difference across the pericanalicular zone of a frog oxyntic cell secreting 0·12 M-hydrochloric acid is c. 6·3 (calculated from Davies & Roughton, 1948), the p.d. between the two sides of this zone, measured with respect to a reference electrode, must be about 370 mV. This p.d. is measurable by electrodes sensitive to H^+ ions only (e.g. glass electrodes). Since the p.d. occurs across a distance not greater than 1 μ (Fig. 9*b*), the electric field is at least 3700 V./cm. between the canaliculus (positive) and the cell wall (negative). If the hypothesis presented above be true, this p.d. due to the pH difference during acid secretion must be more than compensated by that due to the difference

in H atom concentration. Assuming that the H atoms are in equilibrium with hydrogen molecules, then electrodes sensitive to both H_2 and H^+ ions (i.e. hydrogen electrodes), if used together with a reference electrode, would detect a p.d. in the opposite direction, from cell wall (positive) to canaliculus (negative). On this hypothesis the ratio of the equivalent hydrogen pressures must be more than $10^{2 \times 6 \cdot 3} = 10^{12 \cdot 6}$ (see MacDougall, 1939). Such ratios certainly occur within living cells (Hewitt, 1936; Ball, 1942).

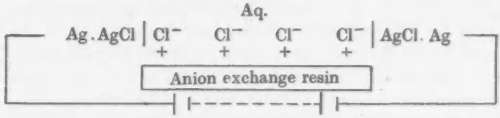
There has been controversy (see Korr, 1939) as to whether redox potentials can be the direct source of bioelectric potentials measurable with non-metallic, i.e. calomel, electrodes. This has been resolved by Korr (1939), who pointed out that the redox potential of the following system can be observed by calomel electrodes



and that if a system able to transfer electrons existed in the cell (as we postulate for the oxyntic cell) then redox potentials could be manifested as bioelectric potentials. Stiehler & Flexner (1938), using indicators, measured the difference in redox potential in the choroid plexus and found it to be 230 mV. with epithelium positive to stroma. Similar results were obtained by Friedenwald & Stiehler (1938) for the ciliary body. It is of interest that, on the basis of these and other results, they suggested that these redox potentials can do secretory and electrical work.

Production of current by tissue. The production of electric power by living tissue is a major problem in itself. If the secretory units in the pericanalicular zone of the oxyntic cells function by the mechanism proposed above, they do not produce a current when actually secreting acid, but can do so when there is no *net* production of H^+, OH^- (and hence HCO_3^-) ions. In this case the H transport system is modified and the H^+ ions, instead of passing into the canaliculus, return and react with OH^- ions to re-form water. This part of the hypothesis is very similar to the 'osmotic diffusion pump' theory published by Franck & Mayer (1947).

The transport of Cl^- ions by the non-acid-secreting cell involves a net transfer of charge, the circuit being completed by conducting pathways inside and outside the mucosa. It can be observed as an electric current through a circuit external to the mucosa, in which the negative sign of the secretory side is determined by the morphological structure of the tissue (Fig. 9*a*). Experiments are in progress in an attempt to determine whether the current consists of Cl^- ions alone, as in the system



or whether cations move in the opposite direction. In this latter case the mucosa must be able to move cations such as H^+, Na^+, etc., from the secretory to the nutrient side.

Potential differences observed. In our present technique we measure the p.d. across the whole mucosa, with saturated calomel electrodes connected to the nutrient and secretory solutions by saturated potassium chloride bridges. It seems likely that the average value of 30 mV. observed across non-secreting mucosa is less than the average p.d. across the pericanalicular zones theoretically observable with calomel electrodes, because of conducting pathways within the mucosa (see Fig. 11*a*). For reasons already given, we consider it probable that the redox potential of the Fe^{++}-Fe^{+++} or similar system which transfers the electrons is the source of this p.d. across the pericanalicular zone. Each secretory unit contributes to the p.d. measured, and there are probably of the order of 10^{13} secretory units in each cm.^{2} of frog gastric mucosa (Crane & Davies, 1947*b*). This figure is based on the known structure and activity of the tissue, and on an assumed turnover number of 10^3 H^+ ions/secretory unit/min. (see McIlwain, 1946). As each secretory unit commences to secrete acid (Fig. 11*b*), then according to the hypothesis there is no longer any net transfer of charge through it, i.e. it is unable to produce a current. Since fewer non-secreting units are now available to maintain the p.d. across the mucosa, whilst the other conducting pathways remain, the p.d. observed externally should fall as more and more secretory units start to produce acid. The experimental results show that such a fall in p.d. occurs. As previously suggested, in the secreting unit the internal electron current, the magnitude of which is affected by the increased metabolic activity of the oxyntic cell, is effective in elaborating the acid secretion, but for reasons given above does not produce a p.d. across the mucosa.

Changes in electric resistance. In non-secreting units the production of a Cl^- ion is believed to be concomitant with the transfer of an electron across the Fe^{++}-Fe^{+++} system. Since during the remainder of the electron cycle there is no net production of H^+ ions, the process is likely to have a low energy requirement and be easily increased or decreased by externally applied electric p.d., i.e. the non-acid-secreting unit will have a low electric resistance. As has been discussed above, the acid-secreting unit produces hydrochloric acid but not current as a result of the cyclic movement of electrons. The same externally applied electric p.d. can now send very little current through the secreting unit, because the Cl^- ions forming the current must be forced across the Fe^{++}-Fe^{+++} system independently of, and in addition to, the electron cycles. Thus the observation of an increase of electric resistance of the mucosa on secretion is in accordance with the hypothesis.

Effect of applied currents. The increase in the rate of acid secretion by the action of applied direct electric currents enhancing the natural p.d. and the decrease by opposing currents (Crane *et al.* 1946; Davies *et al.* 1947; Crane & Davies, 1947*a*; Rehm, 1945) has been mentioned earlier. On the basis of our hypothesis an applied p.d. from cell wall (positive) to canaliculus (negative) could act in two ways, by assisting the return of electrons in the Fe^{++}-Fe^{+++} or similar system, and by assisting the removal of newly formed H^+ ions from the canalicular end of the H transport system, thus speeding up their formation according to the law of mass action. It seems likely that the effect of the applied p.d. on the Cl^- ions would be approximately balanced by its effect on the HCO_3^- ions travelling in the opposite direction. An opposing current would have an effect on the system opposite to that of an enhancing current. A quantitative account of the effects of applied current will be given in a subsequent paper.

An inorganic system which can produce hydrochloric acid by an electron-cycle mechanism. A 'model' of the oxyntic cell has been made which can carry out many of the reactions believed to occur in the living cell.

The system contains two units *A* and *B*. Unit *A* consists of two beakers of dilute NaCl solution (1 and 2) connected by a NaCl bridge and an inverted **U** tube, containing H_2, covering two bent Pt black electrodes. A stream of CO_2 bubbles through solution 1. The pH of each solution is measured with glass electrodes and the whole unit is placed on a small trolley which can move to and fro and, by producing waves, cause the NaCl solutions and the H_2 to cover the Pt electrodes alternately. Unit *B* consists of two beakers, one of $FeCl_2$ (3) and one of $FeCl_3$ (4) connected by an inverted **U** tube packed with the chloride of an anion exchange resin (De-acidite B) which can pass a current consisting virtually of Cl^- ions only. Pt black electrodes connect the solutions with those of unit *A*. A battery can be connected across unit *B*.

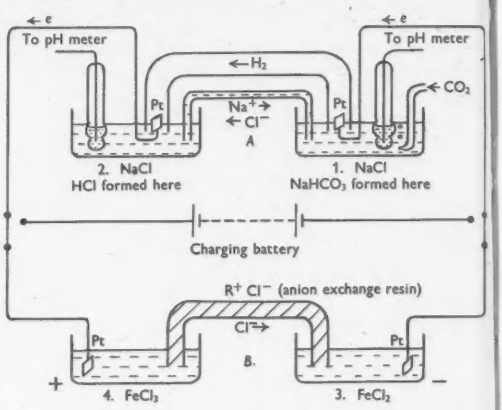


Fig. 12. Diagram of an inorganic system which can produce HCl by an electron-cycle mechanism.

The reactions in unit *A* are:

In 1, $2H^+ + 2e \rightarrow 2H \rightarrow H_2$
$2OH^- + 2CO_2 \rightarrow 2HCO_3^-$.

In 2, $H_2 \rightarrow 2H^+ + 2e$.

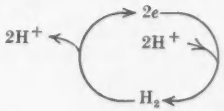
Na^+ ions migrate from 2 to 1 and Cl^- ions from 1 to 2. In unit *B* the reactions are:

In 3, $2Fe^{++} \rightarrow 2Fe^{+++} + 2e$.

In 4, $2Fe^{+++} + 2e \rightarrow 2Fe^{++}$.

Cl^- ions migrate from 4 to 3.

The fundamental overall electron cycle is thus



In this model the reactions stop when the p.d. due to the pH differences in unit *A* counterbalances the redox potential of unit *B*. It seems probable that in the oxyntic cell this process is driven by the differences in hydrogen atom concentration maintained across the secreting unit: it is technically easier in this model to drive the process by the 'electron pressure' difference across the Fe^{++}-Fe^{+++} system which can be recharged with a battery. The maximum *concentration* of hydrochloric acid obtainable by passing current through unit *A* is about twice the concentration of the salts initially present (Na^+ ions migrate to 1, Cl^- ions to 2). Starting from physiological saline solutions, this unit can produce

acid at any concentration claimed for the primary acidity of oxyntic cell secretion (see Babkin, 1944). This is taken to support the electron-cycle mechanism of hydrochloric acid production.

Phenomena accounted for by the electron-cycle mechanism. This electron-cycle mechanism thus fits the following experimental observations on gastric mucosa: the secretion of H^+, Cl^- and HCO_3^- ions, the relations between Q_{HCl}, Q_{O_2} and Q_{CO_2}, and the action of histamine and of carbonic anhydrase; the p.d. across the mucosa and its sign, the decrease of p.d. and increase of resistance on secretion, and the correlation between both Q_{HCl} and resistance changes with p.d. changes; the maintenance of an external current by the mucosa, and the effect of enhancing and opposing currents on acid secretion.

SUMMARY

1. The potential difference (p.d.), resistance, electrical power production and rate of acid secretion of isolated frog gastric mucosa were measured with an apparatus which is described.

2. The secretory side of frog gastric mucosa was negative with respect to the nutrient side in an external circuit. During acid secretion the p.d. was decreased and the resistance increased.

3. Anaerobically the resistance increased and both p.d. and acid secretion were abolished.

4 The mean p.d. across mucosa from the corpus of the stomach was -30 mV.; rectal mucosa -16 mV.; pyloric mucosa -5 mV.; oesophageal, duodenal and ileal mucosa -2 mV.

5. The rise of p.d. after mounting the gastric mucosa was probably caused by the decay of injury potentials. Experiments suggest that the fall in p.d. on secretion was not due to diffusion potentials between the solutions bathing the mucosa.

6. Unstimulated frog gastric mucosae could send a current round an external circuit, producing a maintained electrical power output of the order of 1 μW./mg. dry weight without appreciable polarization. About 10% of the metabolic energy was thereby canalized into electrical energy. This is taken to support the theory that secreting mucosae produce hydrochloric acid by an electrochemical mechanism.

7. Based on the foregoing and other results, an electron-cycle mechanism of acid secretion is suggested, in which H atom and electron transport systems are coupled to produce a net separation of H^+ and OH^- ions in the pericanalicular zone of the oxyntic cells. The energy required is obtained from aerobic metabolism.

We wish to thank Prof. H. A. Krebs, F.R.S., for his encouragement and interest in this work.

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The Effect of Electric Current on HCl Secretion by Isolated Frog Gastric Mucosa

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Hoffmann (1889) was one of the first workers to maintain, on the basis of animal experiments, that stomach secretion was increased by direct current. Many confused and contradictory results have since been published. Claims have been made that direct current affects the motor but not the secretory activity of the human stomach (Schawerin, 1937), the motor and secretory activity (Kirstner, Gorinstein & Rudoy, 1931), and that it has no effect at all (Goldschmidt, 1895). All this early work can be criticized as insufficiently controlled. The secondary effects due to vagal stimulation, when the currents were large enough, were not separated from any direct action on the oxyntic cells.

Recently, Rehm (1945) has shown by controlled experiments on the stomach of a live dog that current passed through the wall of a secreting stomach from mucosa to serosa decreased the rate of secretion of hydrochloric acid, but that current passed from serosa to mucosa increased the rate of acid secretion. In a non-secreting stomach, acid production was not initiated by passing electric current through it in either direction.

These results became available to us during our investigation into the possibility that gastric mucosa could utilize electrical energy presented to it from external sources. Since acid secretion in frog gastric mucosa is accompanied by a change in the electrical potential difference across it, it seemed possible that the rate of secretion might be affected when this p.d. is changed by passing a current through the mucosa.

The experiments described in this paper show that currents enhancing the natural p.d. of isolated frog gastric mucosa increased the rate of acid secretion and sometimes even initiated it. Currents in the opposite direction decreased the rate of acid secretion. The effect was specific to secretive gastric mucosa, i.e. mucosa which is capable of secreting hydrochloric acid (either spontaneously or by stimulation) whether or not it is actually secreting (Crane & Davies, 1947). The significance of the experimental results is discussed.

A part of the present work was communicated to the Biochemical Society (Crane, Davies & Longmuir, 1946).

EXPERIMENTAL

The materials, apparatus and methods used during these experiments were similar to those previously described by Crane, Davies & Longmuir (1948). Three species of frog (*Rana temporaria temporaria* L., *R. esculenta* L. and *R. esculenta ridibunda* Pallas) and toads (*Bufo bufo bufo* L.) were used.

Frog or toad gastric mucosa was mounted in a perspex holder held between two chambers at 25°. The Q_{HCl} of the mucosa (in μl./mg. dry wt./hr.) was estimated from changes in pH of the secretory solution measured *in situ*. In most experiments it was also found by changing the secretory solution and estimating the acid content of the samples thus obtained either manometrically by the addition of a bicarbonate solution, or by electrometric titration using a glass electrode and 0·01 N-NaOH delivered from a Conway burette.

Owing to the buffering capacity of the relatively large amount of mucus secreted by frog gastric mucosa, the HCl concentration determined from the pH of the secretory solution may be considerably less than the total concentration determined manometrically or by electrometric titration (cf. Davies & Longmuir, 1948). In cases where only a pH determination *in situ* was made the value of the acid concentration was corrected for this buffering effect.

The p.d. across the mucosa was measured with a battery-operated Marconi pH meter and potentiometer Type TF 511C, using saturated calomel electrodes and saturated KCl bridges with internal ground-glass joints, which dipped

into the nutrient and secretory solutions. Current was passed through the mucosa by means of zinc-zinc acetate electrodes separated from the solutions bathing the mucosa by the agar layers previously described (Crane *et al.* 1948). The resistance was obtained from the slope of the p.d.-current graph, a correction being made for the resistance of the solution.

The order of procedure was as follows: after the p.d. across the mucosa had become steady, current was passed through the mucosa, usually for periods between 0·5 and 1 hr., alternating with 0·5 hr. rest periods when no current was passed. So that the natural rise and fall of the Q_{HCl} should not be misinterpreted as the result of applied currents, different directions and magnitudes of the currents were used in various orders in consecutive experiments. Errors in the determinations of the change in Q_{HCl} due to a current were in general less than $\pm 0{\cdot}2$ except at low rates of secretion, when accuracies up to $\pm 10^{-4}$ were obtainable. Currents used were usually those giving densities of 0·40 and 1·0 ma./cm.² of mucosa.

RESULTS

It has previously been shown (Crane *et al.* 1948) that the secretory side of isolated frog gastric mucosa is negative with respect to the nutrient (submucosal) side, the mean p.d. across the mucosa being -30 mV. Currents passed through the membrane from nutrient to secretory side increased the magnitude of the p.d. across it and are called enhancing currents. Currents passed in the opposite direction are called opposing currents. A small opposing current decreased the p.d. across the mucosa; on increasing the opposing current the net p.d. became zero and finally increased in the opposite direction. This is shown in Fig. 1, in which the p.d. across the mucosa is plotted

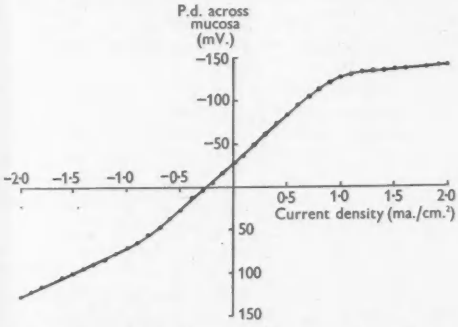

Fig. 1. Relationship between p.d. across an isolated frog gastric mucosa and current density in it. (Slope of curve at any point = resistance of 1 cm.² of mucosa.)

against the applied current density for a typical frog gastric mucosa. The characteristics of this curve will be discussed elsewhere. It is shown here because it formed the basis for the choice of current densities used; 0·4 ma./cm.² was usually below, and 1·0 ma./cm.² usually above, the value at which the change in slope occurred (breakdown current density). Current densities below this breakdown value produced no detectable irreversible change in the mucosa. Current densities just above the breakdown value seemed to be the maximum compatible with subsequent normal functioning of the mucosa.

Gastric mucosae from 28 frogs and 2 toads were used in these experiments, during which current was passed for eighty-four separate periods. Comparable quantitative data were obtained for sixty-one of these periods. Control experiments, which have been described elsewhere (Crane *et al.* 1948), were carried out with 150 other frog gastric mucosae through which no current was passed. Results are described for secretive mucosae when they were initially (*a*) secreting and (*b*) not secreting, for non-secretive gastric mucosae and for six non-gastric membranes.

Secretive mucosae initially secreting acid. In sixteen out of seventeen cases an enhancing current increased the rate of acid secretion from a Q_{HCl} between 0·2 and 2 during control periods to a Q_{HCl} between 1 and 4 while the current was passing. In one experiment the Q_{HCl} was reduced by 0·1 during the passage of an enhancing current of density 0·4 ma./cm.². Histamine had been added to the nutrient solution in six of these seventeen cases. Fig. 2 shows the frequency distribution of the increase in Q_{HCl}, and Fig. 3 the increase plotted against the initial Q_{HCl}. There was no significant difference between the Q_{HCl} increases for the current densities of 0·4 and 1·0 ma./cm.²; the mean increases were $1{\cdot}0 \pm 0{\cdot}3$ and $1{\cdot}2 \pm 0{\cdot}2$ respectively. Figs. 2 and 3 also show the results for opposing currents. In six out of nine experiments the Q_{HCl} was abolished by an opposing current density of 0·4 or 1·0 ma./cm.². In two out of nine experiments 1·0 ma./cm.² reduced the Q_{HCl} almost but not quite to zero; in one case 0·4 ma./cm.² had no apparent effect on a Q_{HCl} of 0·3. Figs. 2 and 3 show further that the absolute change in Q_{HCl} was of the same order of magnitude for both enhancing and opposing currents. The fractional change in Q_{HCl} was greater for enhancing currents; the rate of acid secretion in most cases (twelve out of seventeen) was more than doubled by an enhancing current, whereas in not one case out of nine was it reduced by more than its own value. Such a reduction would correspond to an absorption of acid or secretion of alkali. The mean fractional increase in Q_{HCl} ($\Delta Q_{HCl}/Q_{HCl}$) for the twelve experiments with an enhancing current density of 1·0 ma./cm.² was $2{\cdot}1 \pm 0{\cdot}4$ and for the five experiments with 0·4 ma./cm.² $1{\cdot}4 \pm 0{\cdot}7$. Thus there is no significant difference between the two current densities. The mean fractional decrease of Q_{HCl} for the nine experiments with opposing currents was $0{\cdot}8 \pm 0{\cdot}1$. For all seventeen experiments with enhancing currents the mean fractional increase in

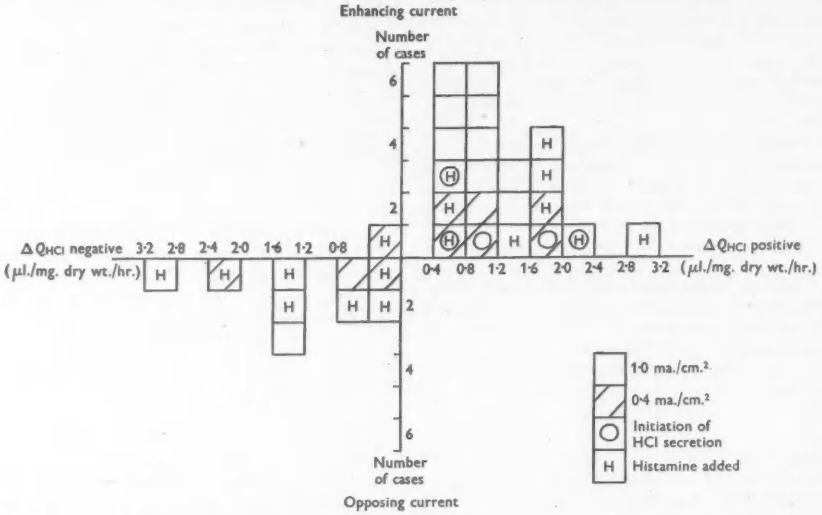

Fig. 2. Frequency distribution of changes in Q_{HCl} due to currents enhancing or opposing the natural p.d. across isolated frog gastric mucosa.

Table 1. *Initiation of hydrochloric acid secretion by enhancing current*

Reference number of mucosa	P.d. level (mV.)	Experimental time: At addition of histamine (hr.) (min.)	Experimental time: At commencement of current (hr.) (min.)	Experimental time: At end of current (hr.) (min.)	Current density (ma./cm.²)	Q_{HCl}: Before current	Q_{HCl}: During current	Q_{HCl}: After current
145	36	—	1 4	2 29	1·0	0·0	0·4	0·0
182	37	—	1 51	2 17	0·4	0·0	1·9	0·6
141	24	1 13	2 35	3 42	1·0	0·0	2·0	2·0
229	28	1 18	3 13	4 29	0·65	0·0	0·4	0·3
229	29	1 18	7 57	9 31	1·0	0·0	0·5	0·3

Q_{HCl} was $1·9 \pm 1·0$; this is barely significantly different from the value for the fractional decrease with an opposing current.

Secretive mucosae not initially secreting acid. Besides the experiments already discussed, Fig. 3 shows five cases (four mucosae) in which the Q_{HCl} rose from zero during the passage of an enhancing current. More detailed results for these cases of apparent initiation of acid secretion by an electric current are given in Table 1. The pH changes of the secretory solution were followed continuously by means of a glass electrode and in all five cases the pH began to decrease immediately after the current was switched on. In one case (no. 141) the Q_{HCl} remained unaltered when the current stopped; in three other cases the Q_{HCl} became smaller and in the remaining case acid secretion ceased when the current was switched off.

On five occasions four other secretive mucosae failed to secrete hydrochloric acid when enhancing

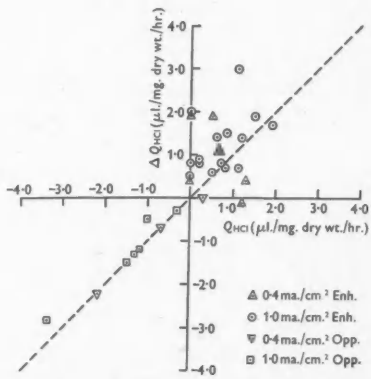

Fig. 3. Relationship between Q_{HCl} and Q_{HCl} due to currents enhancing or opposing the natural p.d. across isolated frog gastric mucosa.

current was passed through them, although they secreted acid at some other time during the experiment. Results for one of these mucosae are shown in Fig. 6; in two others the current was passed before secretion commenced. One of these secreted spontaneously and the other after addition of histamine. The fourth mucosa had finished secreting before the current was passed.

Non-secretive mucosae. Seven mucosae failed to secrete acid at any time during the experiments. Enhancing currents were passed through all these mucosae on sixteen occasions in all, twelve after the addition of histamine. Opposing currents were passed through these mucosae on nine other occasions, six after the addition of histamine. Since the pH of the secretory solution of a non-secreting mucosa was between 6 and 7, its measurement gave a very sensitive method for detecting small hydrochloric acid concentrations. The Q_{HCl} of the above mucosae was less than 10^{-4}; the current densities were usually 0·4 or 1·0 ma./cm.², but current densities up to even 50 ma./cm.² failed to produce acid.

Other membranes. Similar negative results with currents passed in either direction were obtained with all other membranes examined: oesophageal, pyloric, duodenal and rectal mucosa, frog skin, cellophan and dead gastric mucosa. None of these membranes (twenty-three experiments) produced acid (Q_{HCl} less than 10^{-4}) and the alteration of the rate of acid secretion by an electric current thus appears to be specific to secretive gastric mucosa. It is interesting that a mucosa which secreted acid spontaneously and which responded to enhancing and opposing currents failed to secrete acid, even with an enhancing current density of 2 ma./cm.², when its respiration was maximally inhibited by cyanide.

Results for individual mucosae. Figs. 4–8 give examples of the results for typical mucosae. Results of control experiments are shown in Figs. 6–8 of another paper (Crane *et al.* 1948). Fig. 4 shows results for a mucosa in which secretion began after the addition of histamine (5×10^{-5} M) to the nutrient solution. Subsequently an opposing current density of 0·4 ma./cm.², passed through the tissue from secretory to nutrient side, reduced the Q_{HCl} by 2·2. It produced a p.d. of 93 mV. across the mucosa, opposite in sign to its natural p.d. of − 26 mV. After a control period when acid secretion recovered slightly, an equal current passed through the mucosa in the opposite direction, enhanced the p.d. across it to − 148 mV. The Q_{HCl} increased by 1·9 and returned afterwards to a lower value. The resistance of this mucosa was reduced by about half during the passage of the current; the breakdown current density was unusually low and with 0·4 ma./cm.² the resistance was already below its low current value.

Fig. 5 shows the effect of an enhancing current (1 ma./cm.²) on a spontaneously secreting mucosa. The current was passed for three separate periods. During the first two of these the Q_{HCl} was higher than during the preceding and following control

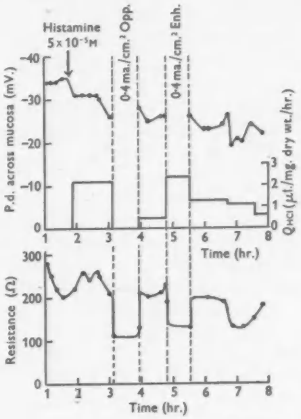

Fig. 4. Effect of enhancing and opposing currents on p.d., Q_{HCl} and resistance of isolated frog gastric mucosa.

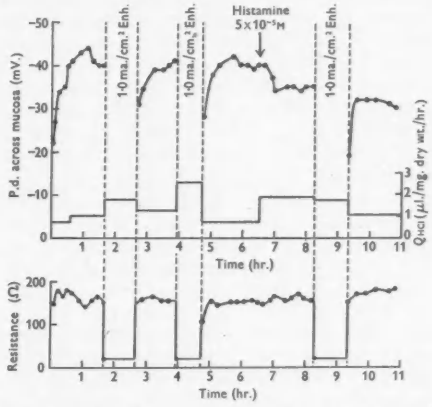

Fig. 5. Effect of enhancing currents (1·0 ma./cm.²) on p.d., Q_{HCl} and resistance of isolated frog gastric mucosa.

periods, and the resistance only one eighth of its normal value. It is interesting that in this case a similar enhancing current after 9 hr. experimental time (E.T.) had no appreciable effect, although the mucosa had responded to histamine at 6 hr. 30 min. E.T. Such anomalous results were observed on some other occasions many hours after mounting.

Fig. 6 shows the behaviour of a mucosa which secreted for two separate periods, once spontaneously

from 2 hr. 30 min. to 3 hr. 30 min. E.T., and again between 5 hr. 30 min. and 9 hr. E.T., after the addition of histamine. On three occasions during secretion an enhancing current increased the Q_{HCl}; but on two other occasions when the mucosa was not already secreting an enhancing current produced no acid whatever.

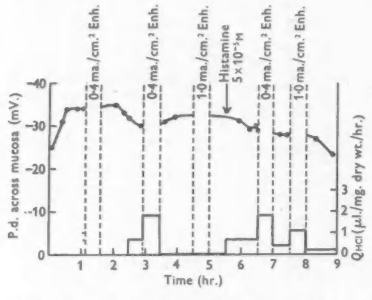

Fig. 6. Effect of enhancing currents (0·4 and 1·0 ma./cm.²) on p.d., and Q_{HCl} of isolated frog gastric mucosa.

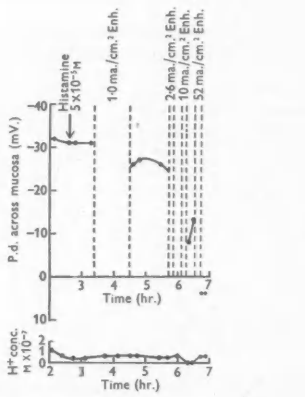

Fig. 7. Effect of large enhancing currents (up to 52 ma./cm.²) on p.d. across non-secretive isolated frog gastric mucosa and on H^+ ion concentration of the secretory solution.

Fig. 8 shows the results of an experiment designed to discover whether mucosa could secrete acid when a p.d. across it was maintained opposite in sign to its natural p.d. An opposing current was switched on at 1 hr. 20 min. E.T. and was adjusted so that the p.d. across the mucosa was 5 mV. in the opposite direction to its natural p.d. of about 30 mV. The reduction of this balancing current during the next 4 hr. suggests that the natural p.d. of the mucosa was

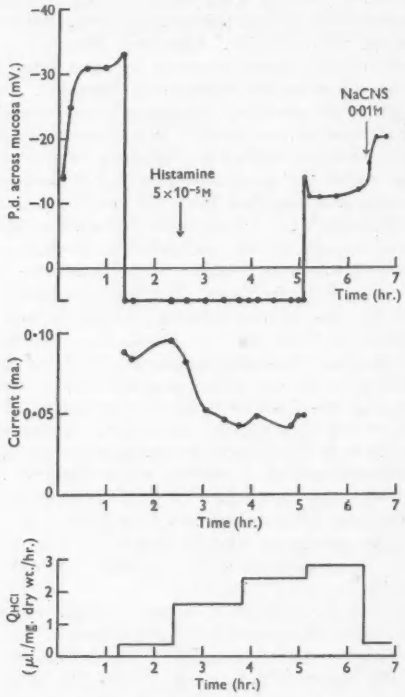

Fig. 8. Secretion with p.d. across isolated frog gastric mucosa maintained at +5 mV. by the application of a current. Effect of 0·01 M-NaCNS on Q_{HCl} and p.d.

Fig. 7 shows results for a typical non-secretive mucosa: an enhancing current density of 1·0 ma./cm.² for over an hour produced no observable effect except that the p.d. was reduced slightly. Subsequent current densities of 2·6, 10 and 52 ma./cm.² also failed to produce any acid (Q_{HCl} less than 10^{-4}), but these high-current densities polarized the mucosa, even reversing the sign of the subsequent p.d. in the case of 50 ma./cm.² Such high currents in either direction could damage secreting mucosa so much that they became permeable to the ions present and the pH of the secretory solution rose.

decreasing during this time, as has previously been found (Crane *et al.* 1948). The secretion curve shows that the spontaneous secretion was increased by the addition of histamine at 2 hr. 30 min. and the Q_{HCl} continued to increase, rising slightly to 2·8 after the current was switched off. The p.d. was now −11 mV. but rose to −20 mV. on the addition of sodium thiocyanate (final concentration 0·01 M) to the nutrient solution, which reduced the Q_{HCl} to 0·4. This substantiates the previous assumption that the p.d. was reduced at the onset of secretion (cf. Crane *et al.* 1948).

DISCUSSION

Polarity of the effect. In the experiments on human patients (e.g. Goldschmidt, 1895; Kirstner *et al.* 1931; Schawerin, 1937) low-current densities were used, less than about 0·5 ma./cm.² of electrode area. The current was passed between parallel electrodes on the dorsal and ventral surfaces of the body at the level of the stomach, so that it was impossible to investigate the effects of currents in different directions through the stomach wall. Moreover, vagal stimulation was not separated from any direct effect on the mucosa.

The experiments of Rehm (1945) on dogs, together with those on isolated frog gastric mucosae presented in this paper, establish the remarkable fact that the rate of secretion of acid by the stomach of a living animal and by isolated gastric mucosa can be altered by the passage of direct current. Since currents from the nutrient to the secretory side of the membrane enhanced the natural p.d. and increased the rate of acid secretion, whilst currents in the opposite direction opposed the natural p.d. and decreased the rate of secretion, the effects cannot be due to stimulation of nerve endings.

These two independent series of results also show that the applied currents do not alter the Q_{HCl} by emptying reservoirs of acid in the mucosa or damming back the acid. If this were the case the raised Q_{HCl} during enhancing current flow would be counterbalanced immediately afterwards by a Q_{HCl} depressed below normal. Similarly, after opposing currents, a Q_{HCl} above normal would be expected. These effects did not occur. An electroendosmotic effect on the secretion of water cannot be the sole cause, since this would merely produce a more dilute secretion containing the same total amount of acid.

In general, frog gastric mucosa appeared to be little, if at all, damaged by the current densities used. Current densities of 0·4 ma./cm.² had no effect on the subsequent p.d. of the mucosa (Fig. 4), but the p.d. was sometimes temporarily reduced for 10–30 min. after current densities of 1·0 ma./cm.² (Fig. 5). A few poor mucosae, however, never recovered their original p.d. after 1·0 ma./cm.². Higher current densities were more injurious; the effect of progressively increasing enhancing currents on the p.d. may be seen in Fig. 7. In general, we confirmed Rehm's results (Rehm, 1945) that high opposing currents caused more damage than similar enhancing currents. A comparison of the relative change in Q_{HCl} in Rehm's and our own experiments is interesting. The examples given by Rehm (1945) (e.g. his Figs. 2, 3A, 3B, 4A) suggest that for 1 ma./cm.² an enhancing current increased the Q_{HCl} by *c.* 20 % and the same opposing current decreased it by *c.* 60 %; whereas in the present experiments the average increase was 210 % and the average decrease 80 % with the same current densities. The gastric mucosa of the dogs used by Rehm (1945) had Q_{HCl} values up to about 50, whilst the isolated frog mucosa used in these experiments had Q_{HCl} values up to about 4.

In Rehm's (1945) experiments the change in Q_{HCl} was found to be roughly proportional to the current used (0–4 ma./cm.²) for both enhancing and opposing currents, the latter being about three times as effective as the former. The present experiments give information only for current densities of 0·4 and 1·0 ma./cm.², but for these the absolute change in Q_{HCl} was not very different, and while the fractional change in Q_{HCl} was 1·5 times as great for the higher as for the lower current density, the difference was not significant. However, the power consumed at these two current densities may be similar. It seems possible that there is a real difference in the ΔQ_{HCl} : current relationships of dog and frog gastric mucosa, but more results are needed.

Specificity. The increased production of acid when enhancing current is passed through secretive gastric mucosa is extremely specific. In twenty-one out of twenty-two cases enhancing current increased the Q_{HCl} of gastric mucosae already secreting acid. In the remaining case ΔQ_{HCl} was $-0{\cdot}1 \pm 0{\cdot}2$, but this is not significantly negative.

It is interesting that while in five out of ten cases enhancing current produced no acid in secretive mucosae not already secreting, in the other five it initiated acid secretion. It is extremely unlikely that spontaneous acid secretion should have chanced to begin in all cases just as the current was switched on (see Table 1). Rehm (1945) found no initiation of acid secretion by enhancing current (density up to 7 ma./cm.²) in any of sixteen cases with live dogs. However, since 'spontaneous' secretion of acid can occur in isolated frog gastric mucosa, but does not occur in the stomachs of live dogs, this difference of behaviour is perhaps not surprising.

The absence of any acid production when current was passed on twenty-five occasions through non-secretive gastric mucosae and on twenty-three occasions through oesophageal, pyloric, duodenal, rectal mucosae, frog skin, cellophan and dead gastric mucosa, strongly suggests that the effect is specific to secretive gastric mucosae, and shows that it is not due to a simple electrolysis. This is confirmed by the absence of any evidence of alkali secretion due to the passage of a current through gastric mucosae or any of the other membranes used.

Significance of the results. Nearly 90 years ago Brücke (1859) suggested that the electromotive forces in gastric mucosa may enable it to send an acid fluid towards the secretory surface and a basic fluid in the opposite direction. Since then many workers (e.g. Engelmann, 1872; Boenheim, 1930;

Keller, 1932) have suggested that glands produce their secretions by electroendosmosis. An attempt was made by Mudd (1926) to prove this hypothesis by applying an electric field, and he believed his data on mammalian serous membranes 'to indicate a probability approaching certainty that electroendosmotic effects are a factor in glandular secretion'.

The first controlled experiments were carried out by Rehm (1945) on the stomachs of live dogs; he has produced much other evidence with this tissue in support of the hypothesis that 'electrical energy provides the energy for the production of osmotic work by living cells' (Rehm, 1943, 1944, 1946; Rehm & Enelow, 1945; Rehm & Hokin, 1947). This work has been confirmed and extended by experiments on isolated frog gastric mucosa (Crane *et al.* 1946, 1948; Crane & Davies, 1947; Davies, Longmuir & Crane, 1947).

It has been shown (Davies, 1948) that, however complex the intermediary processes, the H^+ ions which are elaborated by oxyntic cells come initially from the H atoms in water. In this process electrical work must be done to separate the charges. The experiments of Rehm *et al.* and the present writers, referred to above, together with those recorded in this paper, make it difficult to avoid the conclusion that secreting gastric mucosa can use electric energy from an external source in the production of acid.

Interpretation of the effect. An electron-cycle mechanism of acid secretion has been proposed (Crane *et al.* 1948), in which H atom and electron transport systems are coupled to produce a net separation of H^+ and OH^- ions in the pericanalicular zone of the oxyntic cells. On the basis of the electron-cycle mechanism, the electrical power available from the applied currents and from metabolism will be compared, in a forthcoming paper, with the power needed to produce the observed values of the Q_{HCl}.

SUMMARY

1. Currents up to 50 ma./cm.^{2} were passed through isolated frog gastric mucosa, in directions both enhancing and opposing the natural p.d.

2. In gastric mucosae secreting acid, enhancing currents (from nutrient to secretory side) increased the Q_{HCl}: for 0·4 ma./cm.^{2}, mean $\Delta Q_{HCl} = 1{\cdot}0 \pm 0{\cdot}3$; for 1·0 ma./cm.^{2}, mean $\Delta Q_{HCl} = 1{\cdot}2 \pm 0{\cdot}2$. Similar opposing currents decreased the Q_{HCl} by the same order of magnitude; in many cases secretion was stopped.

3. Four secretive gastric mucosae, not initially secreting acid, commenced to do so when an enhancing current was passed through them; in four other secretive gastric mucosae acid secretion was not initiated by enhancing currents.

4. In all cases currents passed through non-secretive gastric mucosa failed to initiate acid secretion.

5. No other membranes examined (oesophageal, pyloric, duodenal and rectal mucosae, frog skin, cellophan and dead gastric mucosa) produced hydrochloric acid with any currents applied.

We wish to thank Prof. H. A. Krebs, F.R.S., for his encouragement and interest in this work.

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Studies in the Nitrogen Metabolism of the Apple Fruit

CHANGES IN THE NITROGEN METABOLISM OF THE APPLE DURING THE NORMAL AND ETHYLENE-INDUCED CLIMACTERIC RISE IN RATE OF RESPIRATION

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During work carried out over a number of years, it has been found repeatedly that, in Bramley's Seedling apples which have been gathered before the onset of the respiration climacteric and placed in storage, there is a slow rise in net protein content during the period when the respiration climacteric occurs (see, e.g., Hulme, 1936*b*). It is not certain, however, that there is a direct connexion between these two phenomena; it may be that both are changes normally occurring in ripening fruit and that their concurrence may be fortuitous.

Kidd & West (1932) have demonstrated that the respiration climacteric can be induced even in immature fruits by treatment with ethylene. A net rise in protein content, therefore, in relatively immature apples during an ethylene-induced climacteric rise in respiration would be strong presumptive evidence that the two phenomena are intimately connected. The experiments described in this paper were designed, primarily, to ascertain whether this is the case. Data are also given for changes occurring during the 'normal' (i.e. not ethylene-induced) climacteric rise in respiration which occurs eventually even in immature fruits.

Data on the nitrogen metabolism of apples at various stages of development on and off the tree, which have accumulated in this laboratory over a number of years, have suggested that, of the soluble nitrogen fractions presumably involved in protein synthesis, asparagine nitrogen is most closely associated with rise or fall in protein. The present experiments provided an opportunity for studying such a relationship over a short specialized period in the ontogeny of the fruit. The period of the climacteric rise in respiration in immature fruits is especially suitable for such a study, since it embraces a relatively rapid rise in protein under conditions of constant content of total nitrogen.

EXPERIMENTAL

Materials and methods of sampling and storage

Bramley's Seedling apples at two stages of maturity were selected for the experiment. They were taken from 18 trees (18 years old) on East Malling stocks I and II on an unmanured plot at East Malling Research Station. The date of petal fall was 17 May 1937, and the two series of samples were picked on 17 June (Series 1) and 3 August (Series 2). At each pick the requisite number of apples of uniform size was gathered, and each sample was made up from a definite number of fruits from each tree. This procedure ensures that the samples are uniform, each being representative of the group of trees as a whole. *Series* 1 consisted of sixteen samples, each of 22 fruits; the average weight per apple was 16·0 g. *Series* 2 consisted of twenty-one samples, each of 30 fruits; the average weight per apple was 102·5 g. Both series of fruits were, therefore, very immature, the normal commercial date of picking for this variety being about the end of September.

Immediately after preparation, three or four samples were rapidly halved, the seeds removed and the fruits of each sample placed in tins and frozen to $-20°$ to await analysis (Hulme, 1936*a*). These fruits are the 'initial' samples. The remaining samples were each placed in their respective respiration chambers, which formed part of an apparatus for the determination of their rate of respiration in presence and absence of ethylene. This train of apparatus, which was situated in a constant-temperature room at 12°, was similar to that already described (Hulme, 1937), except that a T piece was inserted between the KOH bubbler and the respiration chamber so that ethylene could be introduced into the train at a definite rate through a needle valve and a calibrated bubble bottle. In the case of Series 1 the respiration chamber took the form of a 2 l. Buchner flask in place of the usual vacuum desiccator, and the CO_2-absorption towers were replaced by Pettenkofer tubes.

Preparation of material for analysis and methods of analysis

Separation into peel and pulp tissue and disintegration of the separated tissues was carried out as already described (Hulme, 1936*a*). The seeds of the fruits were collected and their total N content determined so that an indication could be obtained of any possible migration of N between flesh and seed. This factor must be considered, since, in the immature fruits used in these experiments, the seeds were only partially developed and appeared to be still organically connected to the cortical tissue.

Total N was determined on a sample of frozen, ground tissue by the usual Kjeldahl method using 100% (v/v) H_2O_2 as catalyst in addition to $CuSO_4.5H_2O$.

The various N *fractions** were estimated as previously described (Hulme, 1936*a*), the rapid continuous vacuum

* It has been shown (Hulme, 1936*b*) that glutamine is absent from the apple fruit.

extractor (Hulme & Roach, 1936) being used for the 85% ethanol extraction of the tissue.

Titratable acid was determined in the pulp tissue by the method used previously (Hulme, 1936*a*).

Dry weight was determined by drying a sample of frozen ground tissue *in vacuo* at 60° for 48 hr. The results agreed closely with those obtained by exhaustive drying *in vacuo* at 25° over P_2O_5.

Statistical treatment. Replicate samples were taken at intervals as follows: *Series* 1. Four initial samples; two samples at the 15 days' storage point. *Series* 2. Three initial samples; two at 27 days; two at 35 days and two at 55 days. Each series was treated as a separate entity in the statistical analysis of the results. Standard deviations, covering total errors, were calculated from the following equations (Fisher, 1928). Variance $= \Sigma\partial^2$/degrees of freedom, ∂ being the deviation of replicates from their mean. Standard deviation, $\sigma = \sqrt{\text{variance}}$. Statistical analysis of the respiration data was considered unnecessary since determinations were made at very frequent intervals and replicate results agreed to within 2–3 mg. CO_2/100 g./100 hr., even during the period of rapid change associated with the climacteric rise in respiration.

Ethylene treatment

Series 1. The dosage of ethylene in all cases was *c.* 1·5% in the air passing over the treated fruits. This relatively high concentration was used to obtain an immediate response to the introduction of the gas. All twelve samples had been respiring in pure air at 12° for 28 days when the first sample was treated with ethylene; several samples were treated at 36 days, and one final sample at 46 days from zero storage time. Some samples were never treated with ethylene. The time of treatment with ethylene and the time at which treated and untreated samples were frozen to await analysis will be seen from Fig. 1. Where several samples were treated at the same time, the subsequent rate of respiration shown in Fig. 1 is the mean rate of all these samples.

Series 2. Dosage was approximately the same as for Series 1, but all the samples to be treated were given ethylene at the same time, i.e. 20 days after placing in the respiration chambers. The points at which treated and untreated samples were frozen to await analysis are indicated in Fig. 2. The rates of respiration subsequent to ethylene dosage for both treated and untreated samples are the means of the rates for the samples remaining under observation. The number of samples represented by the rate shown in Fig. 2 decreased as samples were removed and frozen.

During the course of the experiments one or two fruits in some of the samples developed infection by moulds. As soon as this was noted the fruit concerned was removed and weighed and the necessary correction applied to the subsequent calculations of rate of respiration.

Table 1. *Apple weight and nitrogen content (per apple) of ethylene-treated and untreated samples, Series* 1, *stored at* 12°; *pulp and peel tissue*

Days in store	Ethylene treatment (+ or −)	Initial fresh wt. of whole fruit (g.)	Dry wt. (mg.)	Total N (mg.)	Protein N (mg.)	Ammonia N (mg.)	Asparagine N (mg.)	Amino-acid N (mg.)	Residual N (mg.)	Titratable acid* (mg.)
					Pulp					
0	−	15·9	1393	12·35	8·70	0·05	2·32	0·32	0·96	256
3	−	17·0	1460	13·72	8·87	0·05	2·96	0·62	1·22	278
7	−	16·1	1338	12·80	7·68	0·05	3·08	1·05	0·93	261
15	−	15·6	1293	13·33	6·55	0·09	4·37	0·81	1·52	238
28	−	15·5	1237	12·50	6·15	0·12	3·92	0·68	1·61	209
36	+	16·2	1248	12·53	7·48	0·09	3·12	0·35	1·49	195
36	−	16·0	1143	12·78	6·80	0·13	3·90	0·54	1·43	199
38	+	15·9	1212	13·80	7·20	0·12	4·40	0·46	1·62	198
41	+	16·1	1164	12·54	7·51	0·11	3·31	0·38	1·23	190
46	+	16·3	1191	13·15	8·11	0·11	3·12	0·52	1·29	183
46	−	16·8	1256	14·09	7·47	0·17	4·26	0·46	1·71	184
64	+	15·3	1024	12·73	8·54	0·11	2·42	0·28	1·18	132
σ	.	1·13	93	0·64	0·40	0·017	0·25	0·058	0·11	21
					Peel					
0	−	15·9	.	4·67	4·14	0·27		.	.	.
3	−	17·0	.	5·03	4·30	0·33		.	.	.
7	−	16·1	.	4·37	3·47	0·44		.	.	.
15	−	15·6	.	4·48	3·19	0·76		.	.	.
28	−	15·5	.	4·30	3·00	0·87		.	.	.
36	+	16·2	.	4·50	3·27	0·84		.	.	.
36	−	16·0	.	4·32	2·79	1·04		.	.	.
38	+	15·9	.	4·69	3·12	1·12		.	.	.
41	+	16·1	.	4·61	3·11	1·08		.	.	.
46	+	16·3	.	4·87	3·50	1·01		.	.	.
46	−	16·8	.	4·81	3·19	1·06		.	.	.
64	+	15·3	.	4·27	3·15	0·81		.	.	.
σ	.	.	.	0·39	0·32	0·049		.	.	.

* Expressed as malic acid.

RESULTS

The primary data for the fruit used and the content of the various nitrogen fractions of the fruit and the total nitrogen content of the seeds during storage with and without ethylene treatment are shown in Tables 1–3. Owing to the small amount of material available, it was only possible to determine total, protein and asparagine + ammonia nitrogen in the peel of the fruit of Series 1.

The levels of significance as between two results in each series of samples (the 'significant difference'), calculated on the basis of a 20% probability ($P = 0{\cdot}05$) are $\sigma \times 3{\cdot}17$ for Series 1 and $\sigma \times 2{\cdot}94$ for Series 2. These values may be approximated to $\sigma \times 3$, so that in both series a difference between two results greater than three times the appropriate standard deviation may be regarded as significant.

An examination of the appropriate significant differences shows that in both series there is no real variation, due to uneven picking, in the weight of the average apple throughout the series and that, as would be expected, there is no significant change in the total nitrogen content during storage. Changes in the fractions of nitrogen are therefore more readily apparent when expressed as a percentage of total nitrogen. The standard deviations for the various nitrogen fractions expressed on this basis are shown in Table 4.

The changes in protein nitrogen, as a percentage of total nitrogen, together with the corresponding changes in rate of respiration are shown in

Table 2. *Apple weight and nitrogen content (per apple) of ethylene-treated and untreated samples, Series 2, stored at 12°; whole fruit (obtained from separate results for pulp and peel) and peel tissue*

Days in store	Ethylene treatment (+ or −)	Initial fresh wt. of whole fruit (g.)	Dry wt. (g.)	Total N (mg.)	Protein N (mg.)	Ammonia N (mg.)	Asparagine N (mg.)	Amino-acid N (mg.)	Residual N (mg.)	Titratable acid* (mg.)
					Whole fruit					(pulp only)
0	−	103·0	14·12	53·62	32·25	0·10	12·17	4·35	4·76	1603
3	−	105·4	14·93	52·00	31·40	0·13	10·92	3·92	5·62	1616
8	−	103·6	14·54	52·68	33·54	0·10	10·07	3·81	5·26	1559
20	−	102·3	13·95	51·96	32·93	0·13	10·02	3·40	5·49	1513
21	+	101·6	13·66	51·50	32·27	0·14	9·50	4·06	5·52	1532
23	+	104·7	13·81	50·18	33·00	0·11	8·75	3·68	4·64	1576
27	+	102·1	13·35	52·39	34·41	0·10	9·54	3·33	5·00	1504
27	−	102·7	13·65	52·22	31·67	0·14	11·72	3·75	4·84	1545
29	−	102·0	13·32	50·85	32·11	0·16	9·94	3·18	5·41	1541
35	−	103·0	13·68	54·27	33·48	0·20	11·98	2·69	5·86	1501
38	−	102·4	13·34	51·50	34·69	0·19	9·44	2·83	4·36	1485
38	+	103·3	13·27	50·54	35·30	0·14	8·05	2·62	4·44	1400
41	−	103·0	13·20	53·94	35·37	0·19	10·92	2·41	5·05	1464
55	−	102·4	13·08	52·69	37·20	0·24	8·54	1·81	4·93	1383
56	+	101·3	12·62	52·66	38·03	0·33	7·81	1·79	4·62	1291
70	−	104·0	12·66	50·63	37·46	0·43	6·40	2·20	4·14	1239
σ	•	1·50	0·34	2·06	1·37	0·017	0·75	0·33	0·39	30
					Peel					
0	−	103·0	2·18	11·96	10·06	0·02	0·83	0·54	0·51	.
3	−	105·4	2·17	11·99	10·22	0·03	0·62	0·50	0·62	.
8	−	103·6	2·32	12·95	10·93	0·02	0·69	0·63	0·68	.
20	−	102·3	2·15	11·96	10·04	0·03	0·66	0·47	0·76	.
21	+	101·6	2·12	11·48	9·63	0·03	0·68	0·44	0·71	.
23	+	104·7	2·09	11·08	9·46	0·02	0·55	0·45	0·60	.
27	+	102·1	2·12	11·89	10·22	0·02	0·63	0·45	0·57	.
27	−	102·7	2·09	11·07	9·04	0·03	0·76	0·43	0·71	.
29	−	102·0	2·09	11·10	9·31	0·03	0·70	0·47	0·59	.
35	−	103·0	2·07	11·33	9·48	0·03	0·77	0·36	0·69	.
38	−	102·4	2·10	11·31	9·83	0·02	0·56	0·43	0·46	.
38	+	103·3	2·06	11·54	10·30	0·03	0·41	0·23	0·57	.
41	−	103·0	2·08	11·67	10·08	0·02	0·66	0·28	0·62	.
55	−	102·4	2·06	11·99	10·79	0·03	0·37	0·23	0·56	.
56	+	101·3	2·01	12·14	10·93	0·03	0·35	0·21	0·62	.
70	−	104·0	2·00	12·03	10·90	0·04	0·30	0·31	0·48	.
σ	•	•	0·030	0·41	0·40	0·005	0·049	0·033	0·052	.

* Expressed as malic acid.

Table 3. *The number, weight and nitrogen content of the seeds of treated and untreated fruits of Series 1 and 2*

Days in store	Ethylene treatment (+ or −)	No. of seeds per apple	Average wt. per apple (mg.)	Average wt. per seed (mg.)	Total N	
					Per apple (mg.)	Per seed (mg.)
			Series 1			
0	−	5·0	117	23	0·57	0·113
3	−	4·5	110	25	0·53	0·120
7	−	4·6	117	25	0·57	0·122
15	−	4·5	96	21	0·44	0·097
28	−	4·4	89	20	0·33	0·075
36	+	4·9	74	15	0·25	0·050
36	−	4·9	72	15	0·23	0·052
38	+	5·1	73	14	0·26	0·049
41	+	4·1	60	14	0·19	0·044
46	+	4·5	63	14	0·21	0·044
46	−	4·2	65	15	0·23	0·051
64	+	4·2	45	11	0·24	0·050
σ	.	0·33	10	2·2	0·046	0·0136
			Series 2			
0	−	4·9	194	40	1·77	0·364
3	−	4·4	181	41	1·69	0·385
8	−	4·8	183	38	1·85	0·385
20	−	4·0	128	33	1·37	0·352
21	+	4·7	166	35	1·70	0·361
23	+	4·8	162	34	1·84	0·383
27	+	4·6	154	33	1·79	0·391
27	−	4·9	144	30	1·59	0·326
29	−	4·5	136	30	1·47	0·328
35	−	4·6	128	28	1·33	0·293
38	−	4·1	123	30	1·57	0·386
38	+	5·2	139	27	1·57	0·300
41	−	4·9	133	27	1·50	0·305
55	−	4·3	121	28	1·57	0·366
56	+	4·3	114	26	1·54	0·355
70	−	.	.	.	.	.
σ	.	0·51	13	1·7	0·147	0·0319

Figs. 1 and 2. With the treated samples the rate of respiration immediately before introduction of ethylene does not fall exactly on the line representing the mean respiration of all untreated samples (continuous line).

Changes in protein in relation to changes in rate of respiration. In view of the fact that there is no significant change in the absolute amount of total nitrogen in the fruit during storage, the results presented in Figs. 1 and 2 indicate clearly that, over the period of the respiration climacteric, whether ethylene-induced or whether occurring as a normal phenomenon in the ontogeny of the fruit, the net amount of protein in both peel and pulp rises significantly. In Series 1 the significant difference for protein nitrogen as a percentage of the total nitrogen is 4·2 for whole fruit and 4·5 for the peel. In all cases the rise in protein nitrogen in the whole apple following ethylene treatment is more than twice the significant difference. In the peel, all the rises are significant except that after the first ethylene treatment; even in this case there is no indication that the rise has ceased when the only treated sample was frozen for analysis. In Series 2, where the significant differences are 2·2 and 2·0% respectively, the rise in protein nitrogen subsequent to ethylene dosage is highly significant for both whole fruit and peel. In this series also the onset of the normal climacteric is followed by a highly significant rise in protein nitrogen in both whole fruit and peel.

It appears, however, that in both peel and pulp tissues of both series the rise in respiration precedes by some hours the rise in protein content (Fig. 1, ethylene treatment at 36 hr. and Fig. 2, both ethylene-treated and untreated samples). The immediate rise in protein following ethylene treatment at 36 hr. in the peel of the fruit of Series 1 is not significant. It is interesting to note that in experiments designed to determine the relative rate of respiration of peel and pulp tissue it has been found (Hulme, unpublished data) that the peel of fruits of 100 g. average weight respires at least three times as rapidly as the pulp tissue. As the present data show, the protein content per unit dry weight of peel tissue is at least twice that of pulp tissue and forms a higher percentage of the total nitrogen (see also Hulme & Smith, 1938).

Changes in the nitrogen content of the seeds. It is clear that the average number of seeds per apple does not vary significantly throughout all the samples (Table 3). The total nitrogen of the seeds

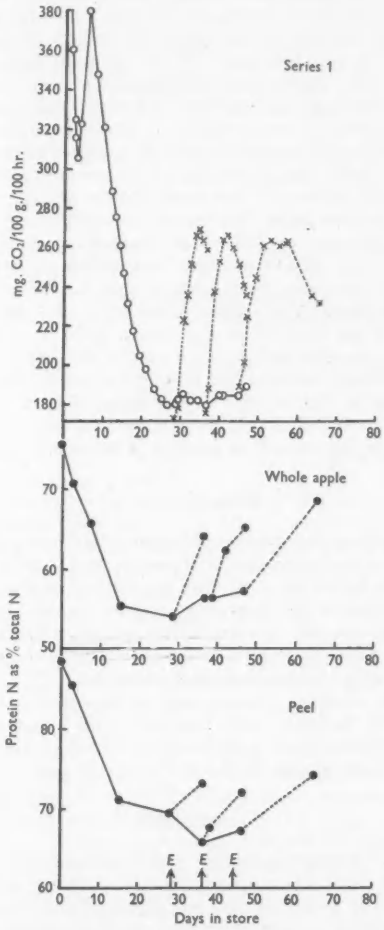

Fig. 1. The change in the rate of respiration (top graph) and the change in content of protein N in the whole apple and in the peel during the storage of apples of Series 1 in air and in ethylene. An *E* above an arrow indicates the point at which ethylene was given to samples whose subsequent history is indicated by an interrupted line.

per apple falls rapidly in Series 1 during the first 36 days of storage, but there is no evidence of any change during the climacteric rise in respiration whether ethylene-induced or not. The fate of the nitrogen lost in the early stages of storage is not

Table 4. *Standard deviations for various nitrogen fractions expressed as a percentage of total nitrogen*

Tissue and fractions	Standard deviation (σ)
Whole apple: Series 1	
Protein N	1·39
Asparagine + ammonia N	1·04
Pulp:	
Protein N	1·78
Ammonia N	0·13
Asparagine N	1·23
Amino-acid N	0·37
Residual N	0·37
Peel:	
Protein N	1·53
Asparagine + ammonia N	0·30
Whole apple: Series 2	
Protein N	0·73
Ammonia N	0·021
Asparagine N	0·97
Amino-acid N	0·81
Residual N	0·40
Peel:	
Protein N	0·67
Ammonia N	0·053
Asparagine N	0·33
Amino-acid N	0·33
Residual N	0·44

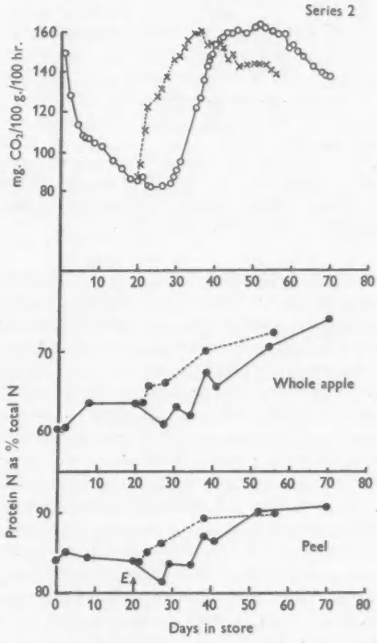

Fig. 2. Changes similar to those depicted in Fig. 1, but for the fruit of Series 2.

clear. In Series 2 there is no significant change in the nitrogen content of the seeds at any time. Thus no transfer of nitrogen from seeds to flesh tissue occurs at any time during the storage of the fruit.

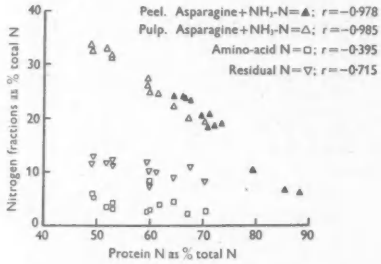

Fig. 3. Relation between protein N and the non-protein N fractions over the whole period of storage of Series 1 fruit, both normal and ethylene-treated.

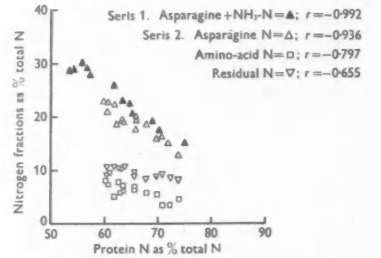

Fig. 4. Relation between protein N and the non-protein N fractions over the whole period of storage of Series 1 and 2 fruits, both untreated and ethylene-treated. The results shown are for the whole fruit.

Changes in the fractions of the non-protein nitrogen during increase or decrease in protein. Since total nitrogen is constant, it may be expected that all the forms of non-protein nitrogen will be affected to some extent by change in content of protein. A cursory examination of the data presented in Tables 1 and 2 suggests that the changes in asparagine nitrogen are most closely related to rise and fall in protein. The relationship between protein nitrogen and the non-protein nitrogen fractions is shown in Figs. 3 and 4. The close correlation between rise in protein nitrogen and fall in asparagine nitrogen is striking. Furthermore, the change in asparagine nitrogen per unit protein (or vice versa) is the same for peel and pulp and covers a wide range of protein levels. The values for asparagine nitrogen in Fig. 4, Series 1, are rather high compared with those of Series 2 owing to the inclusion of ammonia nitrogen in the former case. It is clear that, at both stages of maturity examined, a similar relationship exists throughout the fruit between fall in asparagine and rise in protein.

This general close relationship is confirmed on examination of the correlation coefficients for protein nitrogen on asparagine plus ammonia nitrogen, amino-acid nitrogen and residual nitrogen. Asparagine gives a coefficient (r) of almost unity in every case. The correlation coefficients for amino-acid nitrogen and residual nitrogen are also fairly high, the former not consistently so. Little is yet known of the composition of the residual nitrogen, although it is certain that basic amino-acids are present only in small amounts in the apple (Hulme, 1937).

Titratable acid. The determination of titratable acid has been carried out as a matter of routine in the study of the nitrogen metabolism of apples which has been proceeding in this laboratory for some years. The results obtained in the present study are included in the tables, since it is clear that immediately before the onset of the respiration climacteric (normal and ethylene-induced) there is a rise in the amount of titratable acid in these immature fruits. This rise is, however, only significant in the case of the samples of Series 2.

DISCUSSION

The above data are insufficient for the development of a theory of the paths of protein synthesis in the apple fruit; the most that can be said is that deamidization of asparagine appears to be closely connected with the synthetic processes. Of interest in this connexion is the fact that, while free ammonia nitrogen never forms much more than 1% of the total nitrogen, it does tend to rise significantly during active protein loss and when the rate of protein synthesis is slowing down (Series 1 at day 28; Series 2 towards the end of the storage period).

SUMMARY

1. Evidence is produced that during the climacteric rise in rate of respiration in immature apples, whether occurring normally or whether induced by treatment of the fruit with ethylene, there is a highly significant rise in the net protein content of the fruit.

2. A strong negative correlation is shown to exist between change in protein content and change in asparagine content, not only during the rise in protein which accompanies the respiration climacteric, but also during the steady fall in protein content which precedes the respiration climacteric in very young fruits. The other forms of soluble nitrogenous constituents show some negative correlation with change in protein content, but the correlation is less consistent.

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The Algae

4. THE LIPOCHROMES OF THE MALE AND FEMALE GAMETES OF SOME SPECIES OF THE FUCACEAE

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Biochemical work done hitherto on the algae has been concerned chiefly with the vegetative thallus. Mainly because of the difficulties of securing pure material in sufficiently large quantities, very little consideration has until recently been given to the pigmentation of the reproductive bodies. Among the lower plants, the male gametes of many algae are known to be yellowish in colour, in contrast to the rather more green female gametes, and this difference is especially marked in heterogamous and oogamous plants.

The members of the family Fucaceae (class Phaeophyceae), so abundant on many parts of our British shores, reproduce oogamously by means of minute motile, biciliate sperms and larger non-motile eggs. The dioecious members of the Fucales are especially convenient for study, in that large quantities of both male and female gametes can be obtained in a pure state and free from any possibility of contamination by gametes of the other sex. In the same family, monoecious species also occur, and thus provide material for comparison with the dioecious types.

In the Fucaceae, the antheridia and oogonia are produced within flask-shaped, sunken cavities, the conceptacles. These occur at the periphery of somewhat swollen and mucilaginous receptacles, which in some species are modifications of some of the dichotomies of the thallus as in *Fucus serratus*, *F. vesiculosus* and *F. spiralis*, or in other genera, of the apices of special lateral deciduous fruiting bodies, as in *Ascophyllum nodosum*.

The gradual extrusion of groups of mature eggs and sperms through the openings (ostioles) of the conceptacles, normally occurs between the tides, and is probably brought about in part by the expansion of mucilage, together with contraction on desiccation. In nature, the gametes are removed from the surface of the conceptacles by the incoming tide and fertilization thus takes place outside the parent plant.

The numerous minute biciliated sperms contain a small yellowish chromoplast, and the mature aggregates of sperms therefore appear orange-yellow, which colour they impart to the entire receptacle. This affords a means of distinguishing the male plants in the dioecious species of the Fuci at the time of gamete discharge. In the preparation of material for the present experiments, however, colour alone was not considered to be a sufficiently safe criterion, and a section of a receptacle of every plant collected was examined microscopically, in order to confirm the accuracy of the separation of the male and female plants. Moreover, plants were selected from all parts of the zone of their distribution on the Aberystwyth shore.

The marked orange colour of the exudate of the male plants of the dioecious members of the Fucaceae in their fruiting season, in striking contrast to the olive-green colour of the female-plant exudate, suggested that the colouring matter of the chromatophores of the male and female gametes might possibly be connected with the reproductive process. It seemed feasible that the light-absorbing

properties of the pigments might be responsible for the motility of the male gametes prior to fertilization of the female egg (oosphere).

Immersion in water of the receptacles of the fruiting male plants collected at low tide produces a bright orange suspension of sperms and antheridia, whilst from the fruiting female plants a suspension of eggs (and oogonia) and copious mucilage is secured. From the fruiting monoecious plants, a mixture of eggs and sperms is obtained, the proportions of each varying somewhat during the fruiting period. In the earlier experiments on *Fucus serratus* the orange exudate was removed by wiping, but this method was later rejected in favour of dipping the receptacles in water. From these suspensions the pigments can be extracted with ether or light petroleum, or better still the suspension can be concentrated by centrifuging or filtration. The suspension obtained in sea water consists almost exclusively of active free-swimming sperms with empty antheridial vesicles from which the sperms have been released. A suspension in fresh or distilled water, on the other hand, consists mainly of antheridia with undischarged gametes which renders the process of concentration easier. From the male exudate an orange solution is thus obtained, containing a pigment which exhibits the normal properties of a carotenoid hydrocarbon or ester, i.e. solubility relationships, absorption spectrum, coloration with antimony trichloride in chloroform solution and adsorption properties on alumina, calcium hydroxide and magnesium oxide.

It has been established that the characteristic carotenoid pigments of fresh brown algae are β-carotene and fucoxanthin (Heilbron, Parry & Phipers, 1935; Carter, Heilbron & Lythgoe, 1939; Heilbron, 1942), while in algae which have been kept in a dry condition the latter pigment is replaced by zeaxanthin (Heilbron & Phipers, 1935). A detailed examination of the pigments of the male exudate of the dioecious brown algae *Ascophyllum nodosum* Le Jol, *Fucus serratus* Linn. and *F. vesiculosus* Linn., has revealed that the predominating orange pigment, unaffected by alkaline hydrolysis, is the hydrocarbon β-carotene, already known to be the typical epiphasic carotenoid of the brown algae. A number of other carotenoid pigments is certainly present in very minute quantities; some of these may be isomeric forms of carotene (Carter & Gillam, 1939; Zechmeister, 1944), but although considerable amounts of algae have been employed (the exudate from 250 kg. of *Ascophyllum nodosum* was extracted) it has not been possible to isolate any other pigment in sufficient quantity even for spectrographic analysis. It is of interest to note that, among the fungi, the small motile male gamete of some species of *Allomyces* (e.g. *A. javanicus*) is known to possess a conspicuous orange globule, whilst the larger female gametes are colourless. Emerson & Fox (1940) have found that this orange colour is due to carotenoid pigments, in particular to γ-carotene along with traces of isomers. Also the thick-walled sporangia produced during the sporophytic phase in the life cycle of this fungus contain a brown pigment of the melanin group and no trace of carotenoids.

In direct contrast to the results obtained with the male exudates our examination of the pigments of the corresponding female exudates indicated that chlorophyll and fucoxanthin, both typical pigments of the brown algae, are almost entirely responsible for the colour of the female gametes. Only traces of β-carotene were detected in the extracts, but the fact that the egg possesses a representative sample of the pigments of the parent plant, even in differing proportions, is not unexpected. The crude concentrated extracts of both male and female gametes were largely contaminated with oily impurities, presumably terpenoid in nature, in which the carotenoids are dissolved in the cell chromatophores. (Such materials have been shown (Haas & Hill, 1933) to be present in many members of the Phaeophyceae, and to be more abundant in species growing high in the littoral zone.) These contaminants, particularly in the case of the male exudate, were undoubtedly responsible for the poor yields of β-carotene obtained in a crystalline form, since chromatographic adsorption failed to separate the pigment from these oily impurities. The quantity of fucoxanthin in the female exudate was considerably less than that of the β-carotene in the male; so small was the amount of the former that only from *Ascophyllum nodosum* could any solid material be obtained.

Resolution of the exudate pigments of the monoecious species *Fucus spiralis* Linn., gave results similar to those of the dioecious members examined, in that β-carotene, fucoxanthin and chlorophyll were all present in the mixed exudate, the carotene presumably arising from the orange sperms and the two latter pigments from the greenish eggs.

EXPERIMENTAL

All melting points are uncorrected. The light petroleum used in these experiments had b.p. range 40–60°. Anhydrous Na_2SO_4 was employed for drying solutions, and all operations were carried out under N_2, evaporations being effected under reduced pressure. Alumina refers to Brockmann's 'special for adsorption'; MgO was always diluted with an equal bulk of Hyflo Super-Cel (Strain, 1934). $Ca(OH)_2$ was activated before use by heating at 130° for 1 hr. Elutions were effected with a 5% solution of methanol in light petroleum except where otherwise stated.

Fucus vesiculosus, male. A concentrate of sperms from 13 kg. of the whole fruiting alga was obtained by allowing the alga to stand for 12–24 hr. in a moist atmosphere, and then washing off the exuded material by dipping into a small volume of water. This treatment of the fruiting plants is reasonably comparable with the intertidal conditions on the shore, when the expulsion of the repro-

ductive bodies from the conceptacles is known to take place. The concentrate was centrifuged to remove as much water and mucilage as possible, and the orange residue then ground with anhydrous Na_2SO_4. Treatment of the moist cake so obtained first with methanol and then with light petroleum gave a solution which contained almost all the pigments of the original exudate. The petroleum-methanol solution was washed with water; the resulting light petroleum solution* was then shaken with an equal volume of 95% methanol, but no hypophasic pigments could be separated. The washed and dried light petroleum solution was adsorbed on a column of calcium hydroxide, the chromatogram being developed with the same solvent. The final chromatogram was essentially similar to those obtained from the exudates from male *Ascophyllum nodosum* and *Fucus serratus* (see later), consisting of a lower orange carotene zone in preponderating amount and a complex series of narrow bands in the upper portion of the column. The lower zone was eluted, and, after washing, the solvent was removed. Crystallization of the red oily residue yielded carotene (9 mg.), which on recrystallization gave crystals of impure β-carotene (7 mg.); absorption maxima in carbon disulphide 517, 484 and 454 mμ.

Ascophyllum nodosum, male. The suspension of sperms and antheridia from some 250 kg. of the fruiting alga was treated by a technique similar to that described under *Fucus vesiculosus*, male, except that the suspension was filtered through coarse fluted filter papers which were then extracted with acetone-light petroleum (1 : 4).

By elution of the carotene zone of the chromatogram and crystallization of the resultant red oil (140 mg.) from benzene-methanol, β-carotene (16 mg., m.p. 178°) was obtained as red leaflets with a greenish metallic lustre; absorption maxima in carbon disulphide 517, 487 and 455 mμ. (Carter *et al.* 1939).

Traces of fucoxanthin and chlorophyll, insufficient for characterization except by their behaviour on a chromatogram, were found in the hypophase.

Fucus serratus, male. The exudate was removed from the apices (5 kg.) by wiping with 'Kleenex' cellulose tissues, which were subsequently extracted with light petroleum, into which solvent the entire pigment was transferred.

Treatment of this solution by the technique already described gave a red oil (150 mg.), which on crystallization yielded β-carotene (3 mg.), m.p. 172°. Absorption maxima in light petroleum 479–80 and 449 mμ.; in carbon disulphide 516 and 484–5 mμ.

The mother liquors obtained after separation of the crude carotene still contained much pigment, which was largely contaminated with oily impurities. In spite of saponification and several readsorptions this material failed to yield any further quantity of crystalline product.

Fucus vesiculosus, female. The exudate from 8 kg. of fresh fruiting alga was treated by the technique developed with male *F. vesiculosus*, with the exception that the Na_2SO_4 cake was extracted first with acetone and then with ether. The combined extracts were washed, the dried solution was evaporated and the residue adsorbed, from benzene solution, on a column of alumina, the chromatogram being developed with benzene. A pale-yellow filtrate passed through the column, this consisting of a trace of β-carotene, which in light petroleum solution exhibited absorption maxima at 477 and 446 mμ. The only pigments adsorbed on the alumina were fucoxanthin and chlorophyll. The reddish brown zone of the former, occurring between two greenish bands, gave on elution the characteristic fucoxanthin blue coloration with HCl. Absorption maxima in carbon disulphide were seen at 506–7, 477 and 446 mμ. A mixed chromatogram with pure fucoxanthin on alumina gave only one zone on development.

Ascophyllum nodosum, female. A concentrate of the exudate from 250 kg. of the fresh fruiting alga was treated in a similar manner to that described for *Fucus vesiculosus*, female, except that the residue obtained after centrifuging was extracted directly and not treated with Na_2SO_4.

A trace of β-carotene was obtained as the epiphasic pigment, while the hypophase yielded chlorophyll and impure fucoxanthin (5·5 mg.). This pigment exhibited the characteristic fucoxanthin coloration on treatment in ether with 2·5N-HCl. Absorption maxima in carbon disulphide occurred at 509, 478 and 453 mμ. (510, 477 and 455 mμ. given by Heilbron & Phipers, 1935).

Fucus serratus, female. The exudate of the sexually active apices (4·5 kg.) was extracted in a manner similar to that described for *F. serratus*, male. No pigment could be extracted from the cellulose tissues with light petroleum, but extraction with ethanol gave a dark-green solution.

A trace of an epiphasic pigment, probably carotene, was present, whilst the main pigments, obtained in the hypophasic fraction, were chlorophyll and fucoxanthin. The latter was largely freed from chlorophyll by chromatography, and the pigment characterized by the blue coloration with HCl. Absorption maxima in carbon disulphide occurred at 508 and 473 mμ. with one at 670 mμ. due to presence of a little chlorophyll.

Fucus spiralis, male and female. A concentrate of the exudate from 18 kg. of the fresh, sexually active alga was obtained and treated as described under *F. vesiculosus*, male. The Na_2SO_4 cake was extracted first with acetone and then with ether, the combined extracts were washed, dried and evaporated and the residue was partitioned between equal volumes of light petroleum and 95% methanol. The dark-green petroleum phase was washed with 95% methanol, then with water and dried, and the concentrated solution adsorbed on a column of $Ca(OH)_2$, the chromatogram being developed with light petroleum. The chromatogram consisted of three uppermost greenish brown zones, then a series of very small bands, while the lower part, containing most of the carotenoid pigment, consisted of a single orange zone of β-carotene. Elution yielded insufficient material for crystallization, but the light-petroleum solution showed absorption maxima at 477 and 447 mμ. (see footnote in previous column). The methanol hypophase was diluted with water and extracted with ether, the ethereal solution was washed, dried and evaporated, and the residue transferred to a small volume of dry benzene. This latter solution was adsorbed on a column of $Ca(OH)_2$ and the chromatogram developed first with benzene and then with ether-benzene (1 : 4). The fucoxanthin formed an orange zone below the greenish zones, and on elution it gave the blue coloration with HCl. Absorption maxima in carbon disulphide 476 and 446–7 mμ.

* This solution, and light petroleum solutions containing β-carotene from the other algae, before crystallization, exhibited absorption maxima at 477 and 447 mμ. while similar solutions in light petroleum (b.p. 60–80°) showed maxima at 480 and 448 mμ. (cf. Carter & Gillam, 1939).

DISCUSSION

The pigmentation of the respective gametes of the various Fuci examined is remarkably uniform. This result is not unexpected since the pigments and sterols of the whole plants of the order are similarly uniform (Carter *et al.* 1939). However, the preponderance of β-carotene in the sperm is unusual enough to promote speculation as to the function of that pigment.

In the Fuci, the sexual reproductive phase occurs at a particular period of the year extending in some species over several months, and in this respect the annual fruiting period is believed to spread over a longer period than in most other algae. The reproductive phase is usually followed by a period of vigorous vegetative growth, but elongation does not occur to any perceptible extent while the reproductive organs are being produced. On their discharge into sea water the sperms are extremely active and motile. The period of activity of the sperms, however, begins within the antheridial walls before final release through the apical pore of the antheridium. It might be expected that the energy expended by the sperm is obtained entirely, or in part, from light energy absorbed by the β-carotene which it contains, but evidence from some preliminary experiments which have been carried out suggests that the light absorbed by the β-carotene of the sperms after discharge has no connexion with the ciliary movements or with the fertilization process itself.

These experiments on the motility of *Fucus* sperms, using light of various wave lengths, suggest that the nature of the illumination affects activity only in so far as the velocity of movement and the length of the period of motility are concerned. There is evidence that the sperm suspension retains its vitality and fertilizing power for a relatively longer period if the activity is lessened, and loses it rapidly if the movement is violent. It seems, therefore, that the sperm is fully equipped with a store of energy at the time of discharge and its capacity for movement is determined by its activity and the rate of utilization of this energy store. This is in agreement with experiments on animal sperm activity, since Cohn (1918) showed that the total carbon dioxide production of animal spermatozoa is the same whether their life be long or short.

The accumulation of carotenoid pigments in the reproductive phase and sexual organs is not unusual, and Murneek (1934) has demonstrated that the concentration of carotenoids reaches a maximum in the leaves of *Cosmos*, *Salvia* and *Soja* at about the time of flowering, after which there is a decrease. He points out a suggestive analogy with animals in which carotenoids are present in various organs and secretions usually associated with reproduction and in which the yellow pigments pass from other parts of the body to the reproductive organs, e.g. poultry and cattle.

Different proportions of pigments are reported in some male and female plants, and it appears (Murneek, 1934) that more yellow pigments are present in the female than the male of certain dioecious flowering plants (*Rhamnus*) and fungi (*Mucor*), whilst Delano & Dick (1937) maintain that for some dioecious plants (willow and white poplar) there is more carotene and chlorophyll in the leaves of the male plant than in the female.

The physiological significance of carotenoid pigments in the plant is far from being understood, but within recent years a most important function of these pigments has been revealed. Picrocrocin, crocin and *cis- trans*-crocetin dimethyl esters have been demonstrated to play a fundamental role both in the copulation of the male and female gametes and in the subsequent sex determination in *Chlamydomonas eugametos* (Kuhn, Moewus & Jerchel, 1938; Kuhn, Moewus & Wendt, 1939; Moewus, 1938).

SUMMARY

The predominating colouring matter of the male gametes of various brown algae has been shown to be β-carotene; fucoxanthin and chlorophyll are the pigments of the female gametes.

We are indebted to the Rockefeller Foundation for a grant in aid of this research. We wish to acknowledge the assistance given by the late Dr C. G. Eliot in the examination of *Fucus serratus*, female, and by Dr H. M. David in the laborious task of collecting material for this investigation.

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Quantitative Micro-analysis of Amino-acid Mixtures on Paper Partition Chromatograms

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The successful separation of amino-acids on paper partition chromatograms (Consden, Gordon & Martin, 1944) naturally led to attempts to employ similar methods quantitatively. The methods described below are presented in a somewhat unfinished state, as external circumstances compelled an interruption of the work. In particular, no attempt has been made so far to devise an analytical method suitable for a normal protein. Only the simpler problem has been attempted of analyzing quantitatively such relatively simple mixtures of amino-acids as can be separated using one-dimensional paper chromatograms. No doubt the method could be applied to more complicated mixtures, but preliminary separations, by paper chromatography or other methods, would be required.

PRELIMINARY EXPERIMENTS

Several methods adapted to micro estimations were investigated. Attempts were made to utilize the colour reaction with ninhydrin upon paper. It was found possible to wash off the colour from the paper with a variety of solvents, but the estimation of colour in a photoelectric microcolorimeter did not give consistent results. These experiments, however, showed that the coloured product consisted of several components whose proportions depended upon the exact conditions under which the colour was developed.

Attempts were next made to utilize the Folin reaction, both the original Folin (1922) technique and the modification of Frame, Russell & Wilhelmi (1943), again using a photoelectric microcolorimeter. Both methods were reasonably satisfactory for such quantities of pure amino-acids as are available on a paper chromatogram, but when amino-acids were washed from the chromatogram, interfering substances, apparently derived both from the paper and the solvents, rendered the results grossly inaccurate.

Nitrogen estimation by the Kjeldahl method was also tried. This method is very attractive theoretically, as it was hoped that after a qualitative analysis the chromatogram could be cut up and a quantitative estimate obtained from the amount of nitrogen in each coloured spot. The technique used was that of Hawes & Stavinsky (1942). The scale of working was reduced by a factor of 10, and satisfactory results were obtained, if the starting material was $(NH_4)_2SO_4$ and the incineration omitted. However, the atmosphere of the laboratory proved to be so heavily contaminated with the nitrogenous solvents used in the chromatography that satisfactory incinerations could not be performed. It was not even possible to determine whether the nitrogen blank of the paper was low enough to render the method feasible.

Titration in glacial acetic acid (Harris, 1935) was next employed. The amino-acid was washed from the paper into small titration tubes and, after evaporating in a vacuum desiccator and dissolving in 20 μl. glacial acetic acid, it was titrated with 0·01 N-$HClO_4$ in acetic acid, using crystal violet as indicator (Nadeau & Branchen, 1935). Excellent results were obtained with pure amino-acids, quantities of 15–30 μg. being titratable with an error of 1%. The paper, however, contained such large amounts of acids and bases, which could not be removed by preliminary washing, that the method could not be used on the material washed from the paper.

To avoid interference with contaminating substances, the relatively specific complex formation of the amino-acids with copper was then tried. The technique used was essentially that of Pope & Stevens (1939), which is a modification of the method of Kober (1912). The amino-acid was added to a suspension of copper phosphate in a phosphate-borate mixture, causing the copper to go into solution as the amino-acid-copper complex. After filtration, the copper in solution was estimated iodometrically. Stoicheiometric results were obtained for almost all the amino-acids, with a ratio of two amino-acid molecules to one atom of copper. It was not found possible to reduce the scale of the iodometric titration to the micro quantities available, though good values on 100–200 μg. of pure amino-acid were obtainable. However, even in this case, reducing material derived from the paper interfered with the iodometric titration and prevented satisfactory estimation of the material derived from the paper chromatograms.

Finally, the polarograph (see Kolthoff & Lingane, 1941) was used to estimate the copper complex, in place of the iodometric titration. This proved to be sufficiently specific to give useful though not highly accurate results. As has already been said, the method has not been fully worked out and it is hoped that further experience will increase the accuracy and the availability of the method for complex mixtures.

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EXPERIMENTAL

Polarographic technique

In the initial stages a simple galvanometer, fitted for visual observation, was used with the dropping mercury cathode. Later, a Cambridge Instrument Company's photographic instrument was used. This is convenient when complete current voltage curves are required, but for routine use, as described below, the simple galvanometer is as satisfactory, since observations are made at one potential only.

The dropping mercury cathode used throughout this investigation had the following characteristics. Height of mercury 62 cm., drop time 2·4 sec., rate of flow 2·70 mg./sec. in N-KCl on open circuit. No attempt has been made to work at a constant temperature, but any given set of measurements has been completed within so short a time that temperature variations have been small. There is, however, an obvious advantage to be gained by working at a constant temperature. The polarograph cells were of two sizes, one 9 mm. internal diameter, 8 cm. long, and the other 3 mm. internal diameter and 3 cm. long; each was fitted with a platinum anode sealed into the bottom. The larger cells required 1–2 ml. and the small ones, which have to be completely filled with solution before the electrode is introduced (to avoid air bubbles), required 0·2 ml.

Throughout, sulphite has been used to remove O_2, and has proved more convenient than H_2 or other gas, particularly with the smaller volume of solution. Since, with our solutions, the step at about 0·0 V. before the Cu step begins has been too steep to permit accurate measurement, the zero of the instrument, with the dropping cathode disconnected, has been employed as the reference point.

Supporting electrolyte

A solution of 50 g. $CuCl_2.2H_2O$ was added with stirring to 200 g. $Na_2HPO_4.12H_2O$ in about 1·5 l. of distilled water, and NaOH was added to pH 9·0. The precipitate was filtered on a large Buchner funnel, washed on the filter with 2% borax solution, and then made up to 1 l. with 2% borax solution. This stock suspension can be kept indefinitely. 5 ml. of this suspension, with 1·70 g. $Na_2HPO_4.2H_2O$ and 2 ml. of the starch solution of Pope & Stevens (1939) were made up to 200 ml. with 2% borax. This suspension, which should be made up weekly owing to growth of moulds, forms the supporting electrolyte for the polarographic measurements. The amino-acid was added to a known volume of this suspension and left for 30 min. or more (it can be left for 24 hr. without further change); 1 or 2 ml. were now added to the polarograph cell and a small knife point (*c.* 2 mg.) of Na_2SO_3 was dissolved by stirring with a glass rod (1 mm. diam. with a bent end) which was rolled between fingers and thumb. The dropping electrode was now put into position and allowed to drop for 2 min. with no current passing. The current at −0·5 V. was then observed at 1 min. intervals until steady for 1 min., and then recorded on the photographic drum. The instrumental zero, the dropping electrode having been disconnected, was then immediately recorded on the same segment of the drum. With the large cells the observation was discarded if the current had not become steady within 15 min., and with the small tubes within 5 min. Usually the current had become steady 3 min. after the electrode had been put in.

The shape and height of the current voltage curve depends upon the composition of the supporting electrolyte. In the absence of starch, two pronounced maxima are shown at about −0·3 and −1·3 V. With about 0·01% of starch, both maxima are suppressed, but the height of the step is not much affected (Fig. 1). With higher concentrations of starch the height of the step is greatly reduced and the shape of the curve changed. With the solution used there is a long straight portion from 0·3 to −1·2 V. Measurements have been made at −0·5 V., which has been arbitrarily chosen within this range.

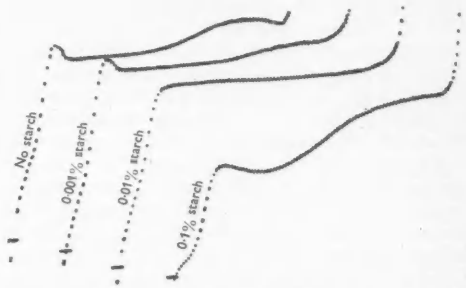

Fig. 1. Effect of starch on the current-voltage curve of the glutamic acid copper complex. The short cross line near the origin of each curve is the 'instrumental zero'.

The amount of Na_2HPO_4 in the supporting electrolyte affects the height of the step. In its absence, copper phosphate is sufficiently soluble to give a large blank of ionic copper. In the presence of high concentrations of Na_2HPO_4 the amount of the ionic copper is so small that an appreciable proportion of the amino-acid does not react, and low results are obtained both polarographically and by iodometric titration. This is true also of a CuO suspension in 2% borax.

The original Pope & Stevens (1939) mixture gave satisfactory results in the polarograph if the solution had been filtered from copper phosphate. If the suspension was used without filtering, at about −0·5 V. the current began to increase, and at higher voltages became very large and independent of the amount of copper in solution. The value of the current in the supporting electrolyte in the absence of amino-acids is very variable. The presence of as little as $2{\cdot}5 \times 10^{-5}$ M-amino-acid greatly reduces the variability. For this reason, no blanks are included in the comparisons given. Perhaps because of the variability it has not been possible to demonstrate a consistent curvature of the current/concentration curve at the lower concentrations of amino-acids. When an amino-acid on a paper chromatogram was to be estimated, the paper containing the amino-acid, which had an area of about 40 cm.^{2}, was folded, cut into pieces of 10 mm.^{2} and introduced into a centrifuge tube by means of a wide-necked funnel; 3 ml. of the supporting electrolyte were then added, which just covered the paper. It was then allowed to stand overnight to ensure that diffusion of the amino-acid from the paper to the copper

phosphate was complete. The paper and the copper phosphate were centrifuged down and the supernatant liquid was transferred to the polarograph cell and the measurement made as before.

Chromatographic technique

The method described by Consden *et al.* (1944) and Consden, Gordon & Martin (1947) has been followed without essential change. Whatman no. 4 paper has, however, been used and HCN has been employed to eliminate the 'pink fronts', where necessary. Wide strips were used, so that the amino-acids would not approach the edge of the paper where irregularity of running is likely to occur.

A sheet of filter paper was marked out with pencil into, say, ten strips, each 4 cm. wide. The solution to be analyzed was applied with a microburette to strips 3, 5, 7 and 9, about 7 cm. from one end. The amount, the same for each strip, should be enough to contain about 20 μg. of each amino-acid to be estimated. To strips 2, 4, 6 and 8 was applied the control mixture, with the amounts on each strip in arithmetical progression, the mean amount being similar to that believed to have been put on strips 3, 5, 7 and 9. Strips 1 and 10 had amounts of control mixture equal to that on strip 8. After development of the chromatogram, and very thorough drying, strips 1 and 10 were cut off, treated with ninhydrin and used as guides for cutting up the other strips. The guide strips have more amino-acid on them than most of the other strips, and hence the separation is probably better on the other strips.

Since there was no direct indication of the proper place to cut, merely an inference from another chromatogram, the line of cutting carried an appreciable error. In order to ensure that the spots are well separated, the time of running of the chromatogram was increased as compared with that employed in qualitative work. If the guide strips show any considerable difference between each other or if the spots are not well separated, that experiment should be abandoned.

In all the measurements a direct comparison has been made between the material under analysis and a control mixture which simulates it as closely as possible. By such a comparison, rather than by attempting an absolute measure, various errors, e.g. those due to variation of temperature or to adsorption by the paper, were minimized.

After the polarographic measurements had been completed in the way described above, the current at 0·5 V. was plotted against the amount of amino-acid for the control series. The best straight line was then drawn (by inspection) through these points (Figs. 8 and 9). This procedure is justifiable over the range of concentration employed, since no consistent curvature has been discovered. The mean of the current readings of the mixture under analysis was then plotted on this line and hence the amount of amino-acid present was inferred.

RESULTS

The results of application of these methods are presented graphically below. Figs. 2 and 3 show the relation between current and concentration of amino-acid, the solution being added directly to the copper phosphate suspension and used without filtration after addition of solid sulphite. No explanation has been found of the variation of both zero current and slope from one experiment to the next, while the results within a given experiment (represented by a single line on the graph) are much more consistent. The copper complex of leucine is too insoluble to be used satisfactorily alone at 10^{-3} M.

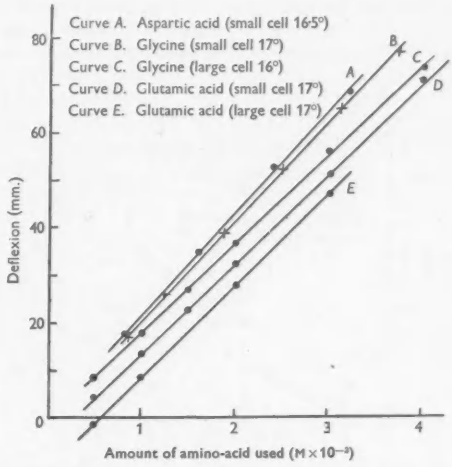

Fig. 2. Relation between current and concentration of amino-acid at −0·5 V. using large and small cells. The zero of the glutamic acid in small cells has been reduced by 5 mm., and glutamic acid in large cells by 10 mm., to avoid overlapping. Sensitivity of galvanometer 1/50.

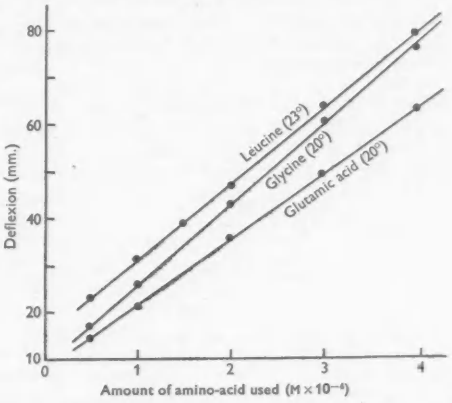

Fig. 3. Relation between current and concentration of amino-acid. The zero of the leucine line has been raised 5 mm. to avoid overlapping. Sensitivity of galvanometer 1/7.

In Fig. 4 a comparison is made between measured amounts of glycine put on a chromatogram, alone and mixed with glutamic acid. The chromatogram was run for 41 hr. in phenol-coal gas-0·3 % NH_3. The extra manipulation and lower concentration has resulted in a decreased consistency of the measurements. Large cells were used here and throughout the remainder of the work.

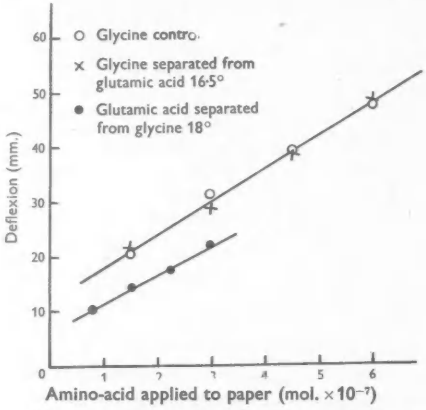
O Glycine control
× Glycine separated from glutamic acid 16·5°
● Glutamic acid separated from glycine 18°
Deflexion (mm.)
Amino-acid applied to paper (mol. ×10⁻⁷)

Fig. 4. Glycine and glutamic acid separated on chromatogram. Galvanometer sensitivity 1/5.

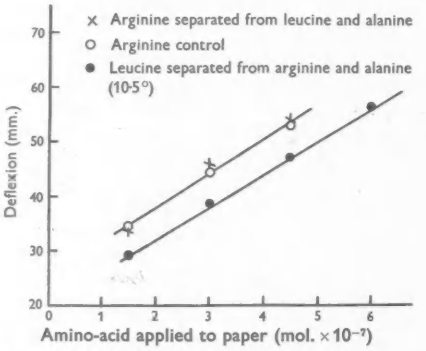
× Arginine separated from leucine and alanine
O Arginine control
● Leucine separated from arginine and alanine (10·5°)
Deflexion (mm.)
Amino-acid applied to paper (mol. ×10⁻⁷)

Fig. 5. Arginine, leucine and alanine separated on chromatogram. Galvanometer sensitivity 1/3.

In Fig. 5 a comparison is made between measured amounts of arginine put on a chromatogram alone and mixed with leucine and alanine. Alanine results were lost. Chromatograms were run 41 hr. in *s*-collidine-diethylamine-HCN.

In Fig. 6 a comparison is made between measured amounts of proline put on a chromatogram alone and as a mixture of equimolar amounts of proline, valine, leucine, glutamic acid, aspartic acid, phenylalanine, tyrosine and ornithine. The chromatogram was run for 67 hr. in *s*-collidine-diethylamine-HCN.

In Fig. 7 a comparison is made between glutamic acid alone and aspartic acid alone and both, in the same mixture as used in the experiment described in Fig. 6. The chromatogram was developed for 67 hr. with phenol-0·3 % NH_3. The aspartic and glutamic acids were not, on this occasion, perfectly separated from each other as judged from the guide strips, though well separated from the six other amino-acids. For this reason the sum of the deflexions for aspartic and glutamic acids has also been plotted for the mixture and the controls.

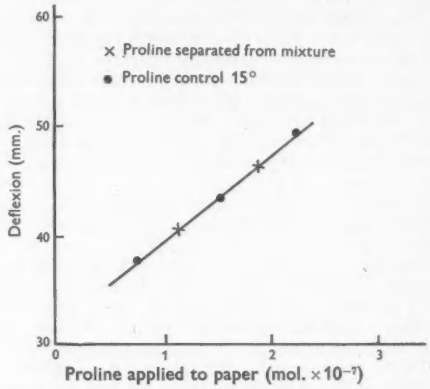

Fig. 6. Proline alone and separated from a mixture. Galvanometer sensitivity 1/3.

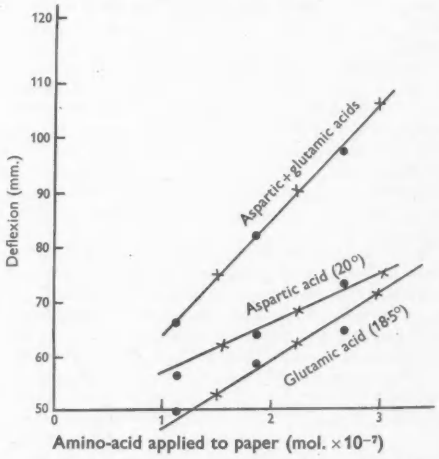

Fig. 7. Glutamic and aspartic acids separated from a mixture of amino-acids. Galvanometer sensitivity 1/2. ×, controls of the single amino-acids; ●, amino-acid applied as mixture.

Analysis of gramicidin S

Figs. 8 and 9 show an analysis of gramicidin *S*. Synge (1945) concluded that this substance consists of equimolecular amounts of ornithine, proline, valine, leucine and phenylalanine, perhaps with 1 or 2 mols. of water.

10·8 mg. of air-dry gramicidin *S* were weighed into a small tube with 1 ml. of 6N-HCl. The tube was sealed and put in an oven at 105–110° for 48 hr. After opening, it was dried in a vacuum desiccator over KOH. To remove further HCl, water was added twice, and evaporated in the desiccator. Finally, 1·013 g. of water were added. 10 μl. of the resulting solution were used on each chromatogram spot. Since altogether nine spots were used, 0·96 mg. of gramicidin *S* was actually consumed in the analysis.

The control mixture contained 12·8 mg. DL-proline, 9·0 mg. DL-valine, 12·7 mg. L-ornithine hydrochloride, 13·3 mg. DL-phenylalanine, 9·8 mg. DL-leucine made up to 10 ml. The ornithine was a rather dark coloured specimen prepared from arginine but free from other amino-acids. The other amino-acids were supplied by Roche Products Ltd. and believed to be pure.

Fig. 8 shows the results obtained with *s*-collidine-diethylamine-HCN as solvent. The chromatogram was run for 67 hr. Comparison was made between the synthetic mixture, run at four different levels, and five lots of gramicidin *S* at the same level of 10 μl. of solution. The deflexions given for the gramicidin *S* were: ornithine, 53·5, 54·0, 54·5, 54·5, 54·5, mean 54·2 mm.; proline, 50·0, 51·5, 51·1, 52·0, 50·0, mean 50·9 mm.; valine, 59·5, 59·0, 59·8, 59·0, mean 59·3 mm. (one lost through cracking of centrifuge tube). The mean values are plotted on the lines given by the control mixture.

Fig. 9 shows results obtained with benzyl alcohol-HCN. The chromatogram was run for 41 hr. Comparison was made between five levels of control mixture and four spots of 10 μl. of gramicidin solution. The deflexions given for the gramicidin *S* were: leucine, 39·0, 39·0, 39·5, 38·5, mean 39·0 mm.; phenylalanine, 36·0, 35·5, 35·0, 35·6, mean 35·5 mm. The mean values of the gramicidin *S* deflexions were plotted on the lines given by the control mixture.

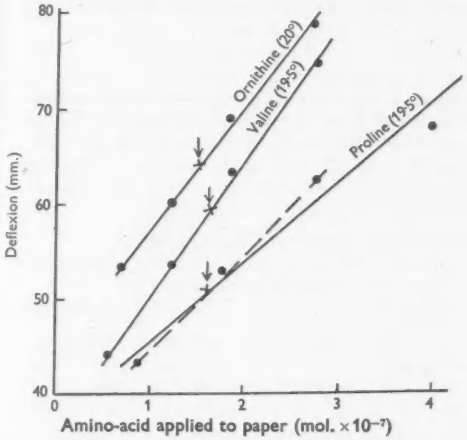

Fig. 8. Analysis of gramicidin *S*. ×, mean of five determinations on 10 μl. of hydrolysate; other points single values of control mixture. Sensitivity of galvanometer 2/3.

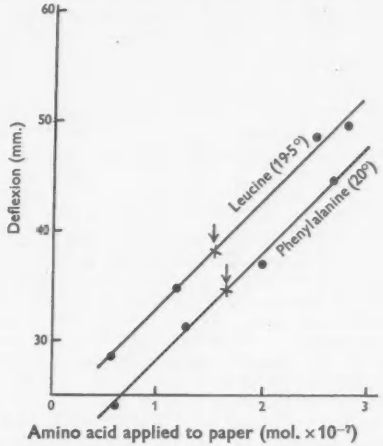

Fig. 9. Analysis of gramicidin *S*. ×, mean of four determinations on 10 μl. of hydrolysate; other points single values of control mixture. Sensitivity of galvanometer 2/3.

From Synge's formula, the mols. of each amino-acid contained in 10 μl. of the hydrolysate are $1{\cdot}69 \times 10^{-7}$ assuming 1 mol. H_2O, and $1{\cdot}64 \times 10^{-7}$ assuming 2 mols. H_2O. The amounts of amino-acids determined from Figs. 8 and 9, in terms of mols. $\times 10^{-7}$, are: Found: proline, 1·62; valine, 1·65; ornithine, 1·55; leucine, 1·53; phenylalanine, 1·67. Expected for all amino-acids: 1·64 mols.

DISCUSSION

An advantage of the use of the lower concentrations of amino-acids which are described here, compared with those used by Kober (1912) or Pope & Stevens (1939), is that amino-acids forming relatively insoluble copper complexes, e.g. leucine and phenylalanine, can be used without the addition of a solubilizer such as glycine or glutamic acid, the use of which is, because of the large amount which has to be added, attended with a large increase of error. Colorimetric methods of measuring copper are available which could almost certainly be made to work at the micro level required, but the labour involved in these is great compared to the use of the

polarograph directly on the suspension. Apart from its use for amino-acids, the method should be applicable to peptides, provided authentic specimens of these exist for comparison. For the amino-acids, the value of the method lies principally in the small amount of material required for analysis, though at present the whole range of amino-acids have not been dealt with simultaneously. For certain amino-acids the microbiological methods, while using comparable amounts of material, are more accurate. For peptides it is improbable that it will be worth while developing the microbiological methods.

The results obtained in the analysis of gramicidin *S*, though intended primarily as a test of the method of analysis, may be considered to confirm the formula proposed by Synge (1945). The material consumed in the analysis here (0·96 mg.) is about 10 % of that which Synge employed, using partition chromatography of acetamido-acids on silica columns.

SUMMARY

1. Various methods have been investigated in an attempt to render quantitative amino-acid analysis by partition chromatography on paper.

2. A polarographic modification of Kober's method for the estimation of amino-acids as their copper complexes is described. A few micrograms only are required.

3. Estimations of several amino-acids in a number of simple mixtures are presented. An analysis of gramicidin *S* using 1 mg. only, confirms the formula proposed by Synge.

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The Amino-acid Composition and Titration Curve of Collagen

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The amino-acid composition of gelatin has been extensively studied, but until recently little attention has been paid to that of its precursor collagen, the natural protein of skin. Some of the analyses of collagen which have been reported were carried out by methods which are now considered unsatisfactory, and in no case have most of the major component amino-acids been determined in one sample of the protein. Further, most of the analyses have been made on collagens which had been given an alkaline or enzyme treatment, or both, to remove the keratins, elastic fibres and reticular tissue which occur in close association with it, and there is a risk that such treatments will cause some modification of the collagen.

For the present study, a sample of collagen has been prepared with the minimum of chemical treatment, since it was considered that the presence of small amounts of elastic fibres and reticular tissue would lead to less error than the treatments necessary to bring about their removal. Also, it seems doubtful whether it is possible to remove these proteins completely by any of the methods which have been suggested. On this preparation Chibnall and his collaborators (Chibnall, 1946; Macpherson, 1946; Rees, 1946; Tristram, 1946) have determined

the basic and hydroxyamino acids, and the following neutral amino-acids: alanine, leucine, isoleucine, valine, phenylalanine and proline; the present authors have determined total N, amino N, amide N, glutamic and aspartic acids, and methionine.

The acid and basic amino-acid contents may also be deduced from the titration curve of a protein. This procedure has an advantage over analysis for the individual amino-acids in that it gives information regarding the reactive groups, including terminal groups, in the intact protein, and comparison of the results with those obtained by direct analysis can give information regarding the way in which the amino-acids are linked in the protein molecule (Chibnall, 1942; Cannan, Kibrick & Palmer, 1941, 1942).

It must be observed, however, that the titration curve of a fibrous protein only gives an exact indication of the titratable groups when it is determined in the presence of salts (Speakman & Hirst, 1933; Jordan Lloyd & Bidder, 1934; Steinhardt & Harris, 1940). This effect of salt is due in the first place to its influence on the Donnan equilibrium. The effective pH value for the equilibrium

$$R\text{COO}^- + \text{H}^+ \rightleftharpoons R\text{COOH}$$

is the pH value in the protein phase; in the absence of salts this pH value is higher than that in the external solution, except at very low pH values, and so the binding of hydrogen ions is less than that corresponding to the external pH value. It has also been suggested (Gilbert & Rideal, 1944) that stoicheiometric binding of hydrogen ions by fibrous proteins only occurs when some anions are also bound in order to lessen the large potential which would otherwise exist between fibre and solution. The addition of salts, by increasing the concentration of anions, will facilitate this binding, and thus favour stoicheiometric binding of hydrogen ions (see also Steinhardt & Harris, 1940). Similar arguments apply to titration with alkali. The concentration of salt necessary for stoicheiometric binding depends on the protein; with collagen it has been shown to be 0·1 M or greater (Retterova, private communication).

In the present investigation the titration curve has been determined in the absence of salts, and also in the presence of 0·5 M-sodium chloride.

METHODS

Preparation of collagen. The back area of a freshly flayed ox hide was cut into pieces (*c.* 18 × 12 in.), which were washed in a revolving glass drum, first with water and then with 10% NaCl for 30 min. The pieces were left stationary overnight in a fresh NaCl solution, drummed for 30 min. in this solution, washed in several changes of distilled water, and dehydrated with acetone. The grain layer (containing the hair roots, and the greater part of the muscle and elastic fibres) and a thin layer from the flesh side were split off, and the remaining material cut into 1 cm. cubes. The cubes were degreased with three changes of light petroleum (b.p. 40–60°) at room temperature for 6 days, washed with successive changes of distilled water, and dehydrated with acetone. The purified collagen contained ash 0·03, moisture 23·0 and grease <0·1%.

Analyses. Total N was determined by the method of Chibnall, Rees & Williams (1943), and amide N by the method of Bailey (1937) and Lugg (1938). The amino N was determined by the Van Slyke manometric procedure, using an auxiliary reaction vessel as described by Doherty & Ogg (1943). To ensure thorough wetting of the sample, the finely shaved collagen (0·15–0·20 g.) was placed in the extraction vessel with 2 ml. of water, the vessel evacuated while shaking, and left under vacuum overnight. The vessel was re-evacuated immediately before the determination.

Dicarboxylic acids were determined by the method of Consden, Gordon & Martin (1948), and methionine by the method of Baernstein (1932).

Titration curves. Collagen (0·75 g.) was placed in 75 ml. of solutions containing measured volumes of HCl and NaOH in resistance glass vessels at 20°. For the titration curve in the presence of salt, sufficient NaCl was added to give a final Cl^- concentration of 0·5 M in the acid solutions, and a final Na^+ concentration of 0·5 M in the alkaline solutions. After 3 days, in which time equilibrium was known to have been reached, samples were withdrawn for pH determinations, and for titration with acid or alkali using bromocresol purple as indicator. When the final solution was too dilute for titration (between pH 3 and 11) the concentration of acid or alkali was obtained from the pH by the use of blank curves for water and for 0·5 M-NaCl; this avoided the necessity of choosing suitable activity coefficients for the two systems. In the more concentrated solutions, the amount of acid or base bound by the collagen is small compared with that remaining in solution, and larger samples of collagen (2 g. in 100 ml.) were used.

pH values were determined at 20° using a glass electrode assembly mounted in a thermostat (Coates, 1945). pH values above 9·0 were determined with a glass electrode of low sodium error (Dole, 1941).

In determining the amounts of alkali bound, precautions were taken to avoid contamination with CO_2. The collagen was placed in the vessel shown in Fig. 1*a*, and the air displaced with CO_2-free air passed in by the tube *B*. The solution of alkali was then added, the tube *A* closed with a small rubber bung, and *B* by a rubber cap. Samples for titration were taken by inserting a pipette at *A*, carrying a similar rubber bung, and forcing the solution into this pipette by passing CO_2-free air into the vessel at *B*. The titration was carried out in a vessel through which a stream of CO_2-free air was continuously blown. In the determination of pH, the solution was forced up into the electrode vessel in a similar way (see Fig. 1*b*), or, in the case of the electrode system mounted in the thermostat, a sample was transferred by means of a pipette.

The soluble N in some of the solutions below pH 2·0 and above pH 12 was determined by micro-Kjeldahl (Markham, 1942) to give an indication of the extent of decomposition, but no attempt was made to apply a correction, owing to lack of information concerning the origin of this N.

Estimate of errors involved in the determination. Errors may arise from the following causes: (1) Difference in the pH value between the solution inside the collagen and the

external solution. (2) Introduction of water with the collagen, thus increasing the total volume of solution. (3) Binding of water by the collagen, thus decreasing the amount of water available for solution of the acid or alkali. (4) Decomposition of the collagen, or liberation of groups not originally present in the native collagen.

Table 1. *Soluble nitrogen derived from collagen after 3 days' contact with acid or alkali*

In the absence of salt		In the presence of 0·5 M-NaCl	
pH	mg. N/g. collagen	pH	mg. N/g. collagen
1·58	0·14	1·64	0·95
1·68	0·12	1·73	0·85
1·80	0·15	1·85	0·77
—	—	2·00	0·69
—	—	2·10	0·70
12·38	1·54	12·46	1·04
12·59	1·72	12·59	1·28
—	—	12·66	1·21
12·78	1·95	12·71	1·08
12·92	2·22	12·86	1·44
13·16	3·86	13·12	3·08

0·03 mmol./g., and at pH 2·0, 0·003 mmol./g. The fact that the curves show well-defined maxima in the acid and alkaline ranges confirms that the net error must be small.

The soluble N present in the solutions (see Table 1) indicates that decomposition of the collagen was small even in the most acid and alkaline solutions. The chief source of this N is probably NH_3 from hydrolysis of amide groups. Under the conditions of the experiment the greater part of this NH_3 will be titrated with acid, thus compensating for the alkali bound by the freed carboxyl groups, and hence such decomposition will not affect the calculation of the amount of alkali bound by the original collagen.

RESULTS

Amino-acid composition

The collagen, prepared as described, may be considered to be a reasonably pure sample. Of the other proteins and tissues present in the fresh skin, albumins and globulins will be removed by treatment in sodium chloride solution (Kritzinger, 1948), and the greater part of the keratins, elastic fibres,

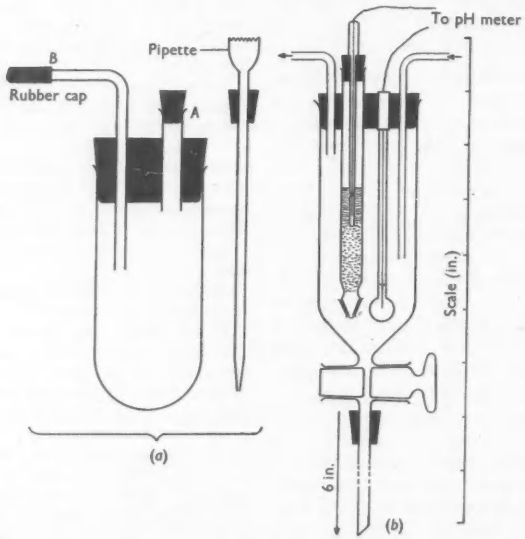

Fig. 1. Apparatus used for the determination of titration curves.

The first of these errors will be appreciable in the absence of salt, and may in part be the cause of the difference in the shape of the curves obtained with and without salt. In the presence of 0·5 M-NaCl it is negligible (Retterova, private communication). Since the second source of error partially compensates for the third, no correction was made for it. As the volume of solution is large compared with the amount of collagen, the net error must always be small. Assuming the collagen binds 50% of water (Cheshire & Holmes, 1942), the error at pH 1 would be of the order of

muscle fibres, sweat glands, fat cells, etc. will be removed with the grain layer (Dempsey, 1946). The remaining impurities are, therefore, a small amount of reticular tissue and some elastic fibres, the presence of which should not greatly affect the results obtained.

The total N of the present collagen preparation is rather higher than the value generally quoted for skin collagen, but is the same as that obtained by

Table 2. *Analyses of collagen*

	N as % protein N	g./100 g. collagen	mmol./g. collagen
Total N	—	18·6	—
Amide N	3·5	0·66	0·47
Amino N	2·5	0·46	0·33
Methionine	0·4	0·8	0·05
Aspartic acid	3·6	6·3	0·47
Glutamic acid	5·8	11·3	0·77

Bergmann & Stein (1939) for ox-hide tendon prepared in a similar manner. Earlier values for skin collagen have been determined on material which has had some treatment (alkaline or enzymic) likely to lead to loss of nitrogen from amide or guanidino groups. The amide N is considerably higher than values quoted for gelatin (Dakin, 1920; Chibnall, 1942), presumably because gelatin is usually made under conditions of alkali treatment which favour the breakdown of the amide groups (Highberger & Stecker, 1941; Ames, 1944). The amino N is of the same order as values obtained by Rutherford, Harris & Smith (1937) and Kanagy & Harris (1935) for hide powder.

The values for the dicarboxylic acids are considerably higher than those obtained by Dakin (1920) for gelatin, and Schneider (1940) for collagen, and are of the same order as values recently obtained for gelatin by microbiological methods (Hac & Snell, 1945; Stokes & Gunness, 1945; Lewes & Olcott, 1945; Hac, Snell & Williams, 1945; Hier, Graham, Friedes & Klein, 1945). Gale (1945), using a specific decarboxylase, found an average value of 10% glutamic acid for this and other similar samples of collagen prepared by the present authors. This value is likely to be low owing to racemization of the glutamic acid during hydrolysis (Gale, 1945).

The methionine content of the present collagen is lower than that quoted by Baernstein (1932) for gelatin, but is in good agreement with recent microbiological determination on gelatin: Horn, Jones & Blum (1946), 0·78%; Lyman, Moseky, Butler, Wood & Hale (1946), 0·88 and 0·83%.

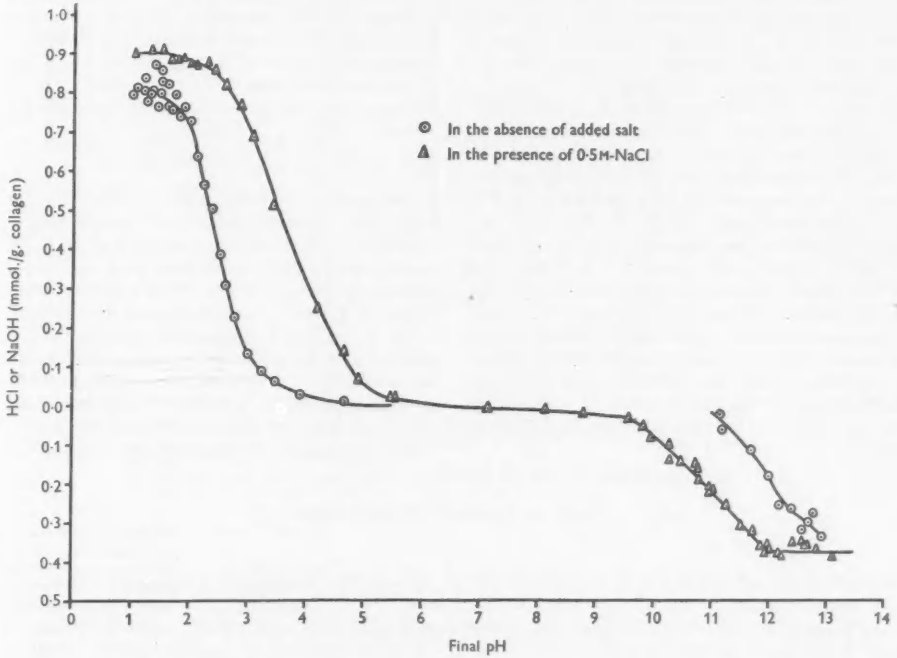

Fig. 2. Titration curves of collagen with and without sodium chloride.

Titration curves

As in previous titration curves of collagen and other fibrous proteins, the curve in the absence of salt shows a broad isoelectric range in which no

combination with acid or alkali takes place, and the acid and alkaline portions of the curve are shifted to lower and higher pH values respectively (Fig. 2). In the presence of sodium chloride, however, there is a definite isoelectric point at pH 6·6–6·8, and the curve approaches that of a soluble protein. The curve shows clearly defined acid and base-binding maxima of 0·90 and 0·36 mmol./g. of collagen at pH 1·5 and 12·5 respectively.

In contrast to the findings of Theis & Jacoby (1941*c*; 1942*b*), who used collagen which had been given a preliminary alkaline treatment, the acid-binding maximum is greater in the presence of salt than in its absence, and there is little indication that the two curves would meet if extended to lower pH values. There is also an indication that maximum base binding is less in the absence of salt.

Previous titration curves of collagen, all of which were determined on collagen which had received either an alkaline or enzymic treatment, show isoelectric points between 5·4 and 7·9 (Theis & Jacoby, 1940, 1941*a*, *b*, *c*; 1942*a*, *b*; 1943*a*, *b*; Atkin, 1937), and in those curves where definite maxima can be distinguished (Theis & Jacoby, 1941*c*, 1942*a*, 1943*a*, *b*), an acid-binding capacity of 0·85–0·90 mmol./g., and a base-binding capacity of 0·38–0·45 mmol./g. The curves of alkali-treated collagen all show an appreciable base-binding capacity in the pH range 6–8, which is absent in the present curve; this is also characteristic of titration curves of gelatin, and may be attributed to increase in the number of carboxyl groups resulting from hydrolysis of amide groups. The present curve can be divided into three portions, pH 1·5–4·9, 4·9–9·6, and 9·6–12·5, and from consideration of the probable pK's of the groups involved, may be interpreted as indicated in Table 3.

It is not possible to distinguish separate sections of the curve due to titration of the imidazole and α-amino groups, as these are present in small amounts and do not differ greatly in pK (imidazole, pK 5·5–6·5; α-amino, pK 7·5–8·5 (Cohn & Edsall, 1943)). Since collagen contains a high proportion of proline and hydroxyproline, it is possible that there are some terminal imino as well as α-amino groups, and these will titrate in a similar pH range (imino of proline, pK 9·7; imino of hydroxyproline, pK 10·1 (Cohn & Edsall, 1943)). It was also not possible to assign any portion of the curve to titration of phenolic hydroxyl groups, and in view of the small amount of tyrosine in collagen and the doubt concerning the extent to which it titrates (Fruton & Lavin, 1939; Steinhardt, 1939; Neuberger, 1943) it has been omitted from the analysis of the curve.

There is no indication that the guanidino group is titrating in the pH range covered, and the pK of this group in collagen must be greater than 14. A similar observation has been made by Lichtenstein (1940) in connexion with the titration curve of gelatin.

DISCUSSION

As far as is known to the present authors, the figures for total N, amide N and amino N are the first to be reported on skin collagen which has received no alkaline or enzymic treatment, and it is not surprising, therefore, that the values for the first two are higher than those generally quoted for collagen.

The free amino N as determined by the Van Slyke method and the number of ε- and α-amino groups deduced from the titration curve (see Table 3) are in close agreement, but appreciably less than the value indicated by determination of the lysine and hydroxylysine in the basic amino-acid fraction of

Table 3. *Analysis of titration curve*

Groups titrating	Method of calculation	pK from curve	Amount present (mmol./g.) From curve	Amount present (mmol./g.) From analysis
(*a*) Total basic groups	Titration from 1·5 to isoelectric point	—	0·90	0·94
(*b*) Imidazole	Titration from 4·9 to 9·6	7·5	0·07	0·05
Imino				—
α-Amino				—
(*c*) ε-Amino*	Titration from 9·6 to 12·5	11·0	0·34	0·39 (0·33 (Van Slyke))
(*d*) Free carboxyl	Titration from 1·5 to 4·9	3·5	0·87	0·79
(*e*) α-Amino + imino	(*b*) − 0·05 mmol. imidazole groups	—	0·02	—
(*f*) Guanidino	(*a*) − (*b*) − (*c*)	>14	0·49	0·51
(*g*) Amide	From analysis	—	—	0·47
(*h*) Dicarboxylic acids	(*d*) + (*g*) − (*e*)†	—	1·32	1·26

* Including the side-chain NH_2 group of hydroxylysine. The pK of the amino group not α to the carboxyl group in hydroxylysine is 9·50 as compared with 10·3 for the ε groups of lysine under the same conditions (Van Slyke, Hiller, MacFadyen, Hastings & Klemperer, 1940), and it may be assumed to titrate in approximately the same range as the ε-amino groups of lysine.

† It is assumed that terminal carboxyl groups are equivalent to α-amino + imino groups.

the hydrolysate (Macpherson, 1946). It is possible that in the intact protein some of the ε-amino groups are bound into the polypeptide chain or for some reason not available for reaction, or alternatively the analytical figure may be too high. The lysine N was determined by difference (total N of basic fraction minus arginine, histidine and hydroxylysine N), and hence is subject to a cumulative error.

The analytical results obtained in this investigation, together with those previously obtained (Chibnall, 1946; Macpherson, 1946; Rees, 1946; Tristram, 1946) on the same sample of collagen, are given in Table 4.

Table 4. *Composition of collagen*

Amino-acid	(1) N as % protein N	(2) As g./100 g.	(3) As g. residues/ 100 g.	(4) As mmol./g.	(5) Minimum mol. wt. calc. from data in (1)	(6) Assumed number of residues	(7) Apparent minimum mol. wt. calc. from (5) and (6)
Total N	—	18·6 *a*	—	—	—	—	—
Amino N	2·5 *a*	0·46	—	0·33	—	—	—
Glycine	26·3 *c*	26·2	19·9	3·50	286	136	38,880
Alanine	8·0 *b*	9·5	7·6	1·06	941	41	38,580
Leucine } Isoleucine }	3·2 *b*	5·6	4·8	0·42	2,350	17	39,950
Valine	2·2 *b*	3·4	2·9	0·29	3,420	11	37,620
Phenylalanine	1·9 *b*	4·2	3·7	0·25	3,960	10	39,600
Tyrosine	0·6 *b*	1·4	1·3	0·08	12,540	3	37,620
Tryptophan	0·0 *d*	0·0	0·0	0·00	—	—	—
Serine	2·5 *b*	3·4	2·7	0·33	3,010	13	39,130
Threonine	1·5 *b*	2·4	2·0	0·20	5,020	8	40,160
Cystine	0·0 *e*	0·0	0·0	0·00	—	—	—
Methionine	0·4 *a*	0·8	0·7	0·05	18,820	2	37,640
Proline	9·9 *b*	15·1	12·7	1·32	760	51	38,760
Hydroxyproline	8·0 *c*	14·0	12·1	1·07	941	41	38,580
Lysine	4·7 *b*	4·5	4·0	0·31	3,200	12	38,400
Hydroxylysine	1·2 *b*	1·3	1·1	0·08	12,560	3	37,680
Arginine	15·3 *b*	8·8	7·9	0·51	1,969	20	39,380
Histidine	1·2 *b*	0·8	0·7	0·05	18,820	2	37,640
Aspartic acid	3·6 *a*	6·3	5·5	0·47	2,092	19	39,750
Glutamic acid	5·8 *a*	11·3	10·0	0·77	1,297	30	38,910
Amide N	3·5 *a*	0·66	—	0·47	2,157	18	38,740
Total found	99·8	119·00*	99·6*	10·76*	—	419	38,730 (mean)

* Excluding amide N.

(*a*) Determined by present authors.
(*b*) Determined by Chibnall and collaborators on the same sample of collagen.
(*c*) Bergmann & Stein (1939).
(*d*) Block & Bolling (1945).
(*e*) Baernstein (1930).

The values obtained by the present authors for the dicarboxylic acids are 50% higher than the earlier values of Dakin (1920), and, although probably still an underestimate, are the best values at present available for collagen. (Consden *et al.* (1948) report 91 and 96% recoveries of glutamic and aspartic acids, respectively, from synthetic mixtures simulating wool.) If a correction based on the results of Consden *et al.* (1948) on wool is applied, the total dicarboxylic acid content agrees well with that deduced from the titration curve.

With the exception of the ε-amino groups of lysine, the values deduced from the titration curve are in agreement with those obtained by analysis, and there is no indication that any of the other reactive side-chain groups of the basic or dicarboxylic acids are bound, or that there is any appreciable number of terminal amino or carboxyl groups.

From a survey of the literature, it is evident that of the remaining amino-acids, tryptophan and cystine are absent, but that glycine and hydroxyproline are present in considerable amounts. Values for these last two have been selected from the literature to complete the attempted survey of the amino-acid composition of collagen given below. Of these, the figure for hydroxyproline is most likely to be in error; the method used is not entirely reliable and the determination was made on gelatin.

When the composition of collagen was considered by Braybrookes (1939) and by Bowes (1943) the analysis was incomplete. The higher figures now obtained for some of the amino-acids, notably phenylalanine and the dicarboxylic acids, and the addition of valine, threonine, serine, and hydroxy-

lysine to the list of amino-acids present in appreciable amounts, have now made up the deficiency. Using the values given in Table 4, it is now possible to account for over 99 % of the total N of collagen (see col. 1), and the sum of the amino-acid residues (col. 3) approaches 100. The analysis of collagen is, therefore, virtually complete. Although the values of some of the amino-acids may be adjusted slightly as a result of improved methods of analysis, it is unlikely that any great changes in the values will be made, or that other amino-acids will be found to be present in important amounts.

Calculation of the minimum molecular weight from the data in cols. 1, 5 and 6 gives a value of about 39,000. Earlier calculations from the composition of gelatin have given figures of 10,300 (Jordan Lloyd, 1920), 10,000–12,700 (Cohn, Hendry & Prentiss, 1925), and 34,500 (Atkin, 1933).

The mean residue weight calculated by summation from the data in Table 4

$$\frac{\Sigma \text{ wt. of amino-acid residues/100 g. protein}}{\Sigma \text{ g. residues/100 g. protein}}$$

is 92·6, and the same value is obtained by calculation from the N distribution (Chibnall, 1942). From X-ray data and the density of dry gelatin Astbury (1940) obtained a value of 94 for the average residue weight in gelatin. Taking 39,000 as the mean molecular weight and 92·6 as the average residue weight, the number of residues/molecule is 421, of which 419 have been identified (col. 6, Table 4).

SUMMARY

1. Collagen has been prepared from ox hide with the minimum of chemical treatment. The total N and amide N contents of the collagen are higher than those previously reported. The glutamic and aspartic acid contents are nearly double the values quoted by Dakin (1920) for gelatin.

2. The titration curve of this collagen has been interpreted in terms of the titratable groups, and the values deduced are, in general, in agreement with those obtained by direct analysis, and indicate that the side-chain groups of the basic and dicarboxylic acids are free, and that the number of terminal groups is small.

3. It is now possible to account for over 99 % of the nitrogen in collagen, and it is considered that it is unlikely that any further amino-acids will be found to be present in important amounts.

4. The minimum molecular weight calculated from the analytical figures is 39,000, and the average residue weight calculated both by summation and from the nitrogen distribution is 92·6.

In the course of this and other work the authors have had occasion to refer to the various determinations of the amino-acids in gelatin and collagen which have been made at different times, and it has been thought useful to put these on record. Copies of this compilation may be obtained from the Librarian, British Leather Manufacturers' Research Association.

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The Effect of Alkalis on Collagen

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An essential process in the conversion of hides and skins to most types of leather is the removal of the hair or wool by treatment in suspensions of lime and sodium sulphide, usually in the cold, for periods varying from a few hours to several weeks.

Although several workers have investigated the effect of mild alkaline treatment on collagen (Marriott, 1928, 1931; Küntzel & Phillips, 1933; Kubelka & Knöedel, 1938; Highberger & Stecker, 1941; Ames, 1944), each has dealt only with one or two of the possible changes which may occur, and in only one instance (Marriott, 1931) has a collagen been used which has had no previous alkaline or enzymic treatment.

The present investigation has been carried out, therefore, on a collagen which has received no previous chemical treatment likely to cause chemical modification (see Bowes & Kenten, 1948), with the aim of obtaining more precise information regarding possible changes occurring during mild alkaline treatment.

EXPERIMENTAL

Alkali treatment of collagen. Collagen prepared as previously described (Bowes & Kenten, 1948) was cut into cubes about 1 cm. square, and 100 g. of this material, together with 310 ml. of water, 1·55 g. of $Ca(OH)_2$, and 90 ml. of N-NaOH were incubated in a sealed flask at 20°. A preliminary experiment showed that these quantities gave an initial OH^- concentration of 135 m-equiv./l. (pH 13·3 at 20°), and a final value of 68 m-equiv./l. (pH 13·0 at 20°). After 14 days the flask was cautiously opened to avoid loss of NH_3, sufficient HCl added to bring the contents to pH 4·5–5·0, the flask resealed and left at 20° for 3 days. Two batches of collagen (I and II) were treated in this way.

The collagen was filtered off, washed in running water for several hours, treated repeatedly with acetic acid pH 3 containing 1·5% NaCl, exhaustively washed with water at pH 8, finally with water at pH 6, then dehydrated with acetone. The pH of the last wash water was 5·5. (A little toluene was added to all wash waters to inhibit the growth of micro-organisms.)

An appreciable amount of protein matter (solubilized collagen) was precipitated on neutralization. This material was filtered off with the collagen and removed during the washing in running water.

The total N, amino N, NH_3 and urea were determined in the solution in which the collagen had been treated. It was assumed that the solution held by the collagen was of the same composition as the external solution, and that the total volume was equal to that of the solutions added plus the water held by the original collagen, and the results were calculated accordingly. The total N, amino N, amide N, arginine, total hydroxy acids (serine + threonine + hydroxylysine), and the titration and swelling curves of the collagen, were also determined. All results are expressed on moisture- and ash-free collagen. Since the total N of the alkali-treated collagen is lower than that of the original collagen, the results expressed as a percentage of the total N may be misleading, and they have therefore been recorded also as mmol./g. protein.

Analysis. Total N, amide N and amino N were determined as previously described (Bowes & Kenten, 1948). Arginine was determined by precipitation with flavianic acid (Vickery, 1940). The value obtained (8·3%) was rather lower than that of 8·8% found for the same collagen sample by Macpherson (1946), who used a colorimetric method following the separation of the basic amino-acids by electrodialysis.

Hydroxyamino-acids. The total hydroxyamino-acids (serine + threonine + hydroxylysine) were determined by the periodate method as described by Rees (1946), except that the periodate oxidation and subsequent distillation of the NH_3 were carried out in a micro-Kjeldahl apparatus. The following solutions were successively added to the micro-Kjeldahl apparatus, while preserving a liquid seal at the ground-glass stopper: 5 ml. of hydrolysate, 5N-NaOH to adjust the pH to 7, N-H_2SO_4 (sufficient to give a final pH of 7·0 after addition of other reagents), 1·5 ml. of $NaAsO_2$ solution, 6 ml. of phosphate buffer pH 7·2, 1 ml. of 0·5M-HIO_4, 3 ml. water. The contents of the micro-Kjeldahl flask were thoroughly mixed after each addition by warming the steam flask, and thereby causing a stream of air bubbles to pass through the solution. The NH_3 (preformed and periodate) was distilled off from 5 ml. of borate buffer pH 12·0. The preformed NH_3 was determined by a separate distillation of 5 ml. of hydrolysate at pH 7·0 from 5 ml. of borate buffer.

Other determinations. Ammonia in solution was determined by addition of excess K_2CO_3 and aerating into standard acid; urea by conversion to NH_3 by the action of urease followed by the same procedure (Van Slyke & Cullen, 1914). The urea NH_3 was then calculated by difference. It was necessary to use twice as much urease as that employed by Van Slyke & Cullen (1914) in order to obtain satisfactory results; this was probably due to inhibition of the urease by the high salt concentration of the solution (cf. Conway, 1947). The amount of urea was small compared with that of NH_3, and tests showed that under the present experimental conditions the method gave an accuracy of ±10%.

Titration and swelling curves. The titration curve of the alkali-treated collagen (batch I) was determined in the presence of 0·5M-NaCl as previously described (Bowes & Kenten, 1948). For the swelling curves, 0·5 g. samples of air-dry collagen were placed in 100 ml. of solution containing varying amounts of HCl and NaOH at 20°. After 3 days, a sample of liquid was withdrawn for pH determination, and the pieces of collagen lightly blotted and weighed. The vessels used were the same as those employed for the titration curve, and the same precautions were taken to avoid contamination with CO_2. The water content, expressed as a percentage of the moisture and ash-free collagen, was plotted against the final pH value of the solution.

RESULTS

Analytical

There is good agreement between the results on the two separate batches of treated collagen, indicating that the results obtained are reproducible (see Table 1).

Although the treatment given was comparatively drastic, the collagen only decreased in weight by about 5%, and was unchanged in appearance. Approximately half the amide N was liberated during 14 days' treatment, and a corresponding amount of ammonia was found in the solution; there

Table 1. *Analyses of collagen, alkali-treated collagen and of collagen dissolved by alkaline solution*

	Collagen					Solution		
		Alkali-treated		Increase or decrease				
	Un-treated	Batch I	Batch II	Batch I	Batch II		Batch I	Batch II
Ash (%)	0·03	0·01	0·10	—	—	—	—	—
Water (%)	22·3	23·7	23·8	—	—	—	—	—
Total N (%)	18·6	18·2	18·1	−0·4	−0·5	Total N (mg./g.)	9·0	8·1
Amino N (as % TPN)	2·48	2·77	2·94	—	—	Amino N (mg./g.)	4·3	4·3
Amino N (mmol./g.)	0·33	0·36	0·38	+0·03	+0·05	—	—	—
Amide N (as % TPN)	3·54	1·69	1·85	—	—	Ammonia N (mg./g.)	3·46	3·36
Amide N (mmol./g.)	0·47	0·22	0·24	−0·25	−0·23	Ammonia N (mmol./g.)	0·25	0·26
Arginine N (as % TPN)	14·45	14·17	14·24	—	—	Urea N (mg./g.)	0·45	0·42
Arginine N (mmol./g.)	0·48	0·46	0·46	−0·02	−0·02	Urea (mmol./g.)	0·016	0·015
Periodate NH_3 (as % TPN)	4·54	4·45	—	—	—	—	—	—
Periodate NH_3 (mmol./g.)	0·60	0·58	—	−0·02	—	—	—	—

TPN = total protein nitrogen.

was no indication that an appreciable amount of ammonia was derived from any other source. The amount of ammonia formed was almost identical with the maximum value obtained by Highberger & Stecker (1941). Further treatment for 7 days under similar conditions, however, lowered the amide N by another 0·08 mmol. to 0·16 mmol./g., with the production of 0·06 mmol. of ammonia.

The small decrease in the arginine content is less than that found by Theis & Jacoby (1941) in similar experiments, and the urea found in the solution was also less than that found by Highberger & Stecker (1941) under corresponding conditions. It appears probable that the urea arises from conversion of the arginine to ornithine residues (cf. Hellermann & Stock, 1938; Warner, 1942*a*); the decrease in arginine (this small increase could be accounted for by the conversion of arginine to ornithine), and, even allowing for the fact that because of the high proline and hydroxyproline content of collagen one out of every four peptide links involves an imino and not an amino group, the alkaline treatment has apparently caused little breakdown of the polypeptide chains of the remaining insoluble collagen.

Titration curves

At pH values between 5 and 10, a longer time was required to reach equilibrium with alkali-treated collagen than with the original collagen. There was rather more soluble nitrogen at high pH values, and rather less at low pH values than with the original collagen (see Table 2).

Table 2. *Nitrogen in solution at different* pH *values after steeping for* 3 *days*

(Results expressed as mg. N/g. moisture- and ash-free collagen.)

Collagen				Alkali-treated collagen			
pH (20°)	Soluble N	pH (20°)	Soluble N	pH (20°)	Soluble N	pH (20°)	Soluble N
1·16	0·85	12·86	1·44	1·18	0·48	—	—
1·32	0·77	12·71	1·08	—	—	12·77	2·15
1·51	0·69	12·66	1·21	1·42	0·36	12·69	2·05
1·64	0·70	12·59	1·28	1·82	0·34	12·54	2·26
—	—	12·46	1·04	—	—	12·39	1·26
—	—	—	—	—	—	12·20	1·45

content, however, is rather greater than can be accounted for as urea, indicating some other type of breakdown. The alkali-treated collagen, when tested for the presence of citrulline by the method of Fearon (1939), gave a very faint pink colour, the original none at all.

Within experimental error, the ammonia formed by the action of periodate on the untreated and alkali-treated collagen is the same, indicating a negligible destruction of the hydroxyamino-acids by the alkaline treatment. The hydroxyamino-acids of the collagen used in this investigation have also been determined by Rees (1946), who obtained a value of 4·57 % for the periodate NH_3 (expressed as a percentage of the total protein N), in good agreement with the present determination of 4·54 %.

The ammonia and urea in the solution account only for about half of the total N present. The figure for the amino N of the solution is likely to be too high owing to the presence of ammonia (25–30 % of which may be returned as amino N (Warner, 1942*b*; Frost & Heinsen, 1945)), but the results suggest that this soluble N must be present as amino-acids or small peptides. It may be observed that the sum of the ammonia and urea N in solution is approximately equal to the decrease in the total N of the collagen.

The amino N of the alkali-treated collagen was only slightly higher than that of the original collagen

The titration curve of the alkali-treated collagen differs in a number of respects from the curve of the original collagen, especially in the alkaline range (see Fig. 1). The isoelectric point is shifted from pH 6·7 to 5, the acid binding is slightly increased, and on the alkaline side of the isoelectric point there is a large increase in the number of groups titrating between pH 5 and 9, and a corresponding increase in the base binding capacity. The large increase in the groups titrating between pH 5 and 9 may be attributed to an increase in the number of carboxyl groups arising from hydrolysis of amide groups.

The curve was analyzed in a manner similar to that of the untreated collagen (see Bowes & Kenten, 1948). It can be divided into four portions, from pH 1·5 to 5, from 5 to 7, from 7 to 9·6 and from 9·6 to 12, and to these different portions are assigned the groups shown in Table 3. Imino groups, due to breakdown of peptide links involving proline or hydroxyproline, are taken as mainly titrating between pH 7 and 9·6.

Assuming that the imidazole groups are unaffected by alkaline treatment (Plimmer, 1916), and that they titrate in the same range as in the untreated collagen, it can be deduced that there are 0·14 mmol. uncharged carboxyl groups/g., and 0·13 mmol. basic groups/g. (α-amino, imidazole or imino), titrating between the isoelectric point and pH 9·6. The assumption that the imidazole groups

are not affected may not be strictly true, since there is some evidence that gelatin contains less histidine than collagen (Chibnall, 1946). The difference, however, is small, and even if a corresponding decrease in histidine does occur here, it would not materially affect the calculation. The same conclusion is reached if the increase in acid-binding capacity is taken as due to increase in the number of α-amino and imino groups, again assuming that these groups titrate between pH 5 and 9·6, and that the α-amino and imidazole groups present in the original collagen remain unchanged.

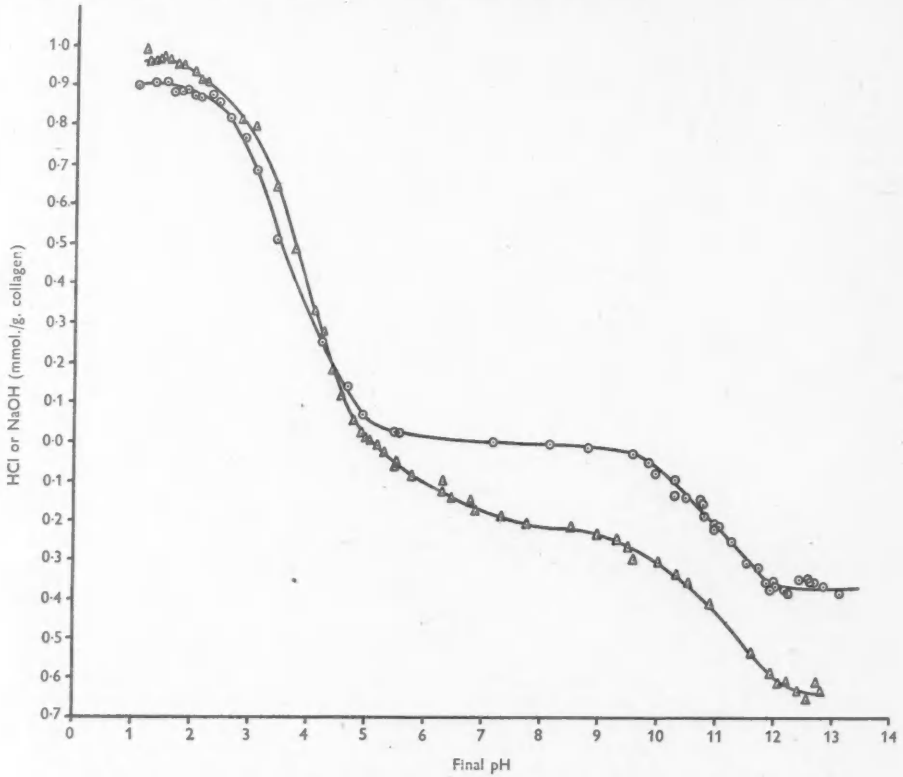

Fig. 1. Titration curves of ox-hide collagen before (circles) and after (triangles) treatment with alkali.

Table 3. *Analyses of titration curves*

(Results expressed as mmol./g.)

Group	Method of calculation	Original collagen	Alkali-treated collagen
(*a*) Charged carboxyl	Titration from pH 1·5 to 5	0·87	0·96
(*b*) Uncharged carboxyl + imidazole	Titration from pH 5 to 7	0·03	0·17
(*c*) Imidazole, α-amino + imino	Titration from pH 7 to 9·6	0·04 } 0·07	0·10 } 0·27
(*d*) ε-Amino	Titration from pH 9·6 to 12·5	0·34	0·36
(*e*) Uncharged carboxyl	(*b*) − 0·03 mmol. imidazole groups titrating in this range	0	0·14
(*f*) Total carboxyl	(*a*) + (*e*)	0·87	1·10
(*g*) α-Amino + imino	(*c*) − 0·02 mmol. imidazole groups titrating in this range	0·02	0·08
(*h*) Guanidino	(*a*) + (*e*) − (*b*) − (*c*) − (*d*)	0·49	0·47
(*i*) Amide	From analysis	0·47	0·22
(*j*) Dicarboxylic acids	(*f*) + (*i*) − (*g*)*	1·32	1·24

* It is assumed that terminal carboxyl groups are equivalent to the free α-amino and imino groups.

If these conclusions are correct, there is an apparent decrease in the dicarboxylic acids, which suggests that there may have been specific loss of these amino-acids in the fraction of the collagen rendered soluble by the alkaline treatment.

The decrease in arginine content calculated from the titration curve agrees well with that found by analysis (Table 1), and the small increase in groups titrating between pH 9·6 and 12 is in accordance with the hypothesis that the arginine is converted to ornithine (pK 10·76) (Cohn & Edsall, 1943). There is no indication of any decrease in the number of ϵ-amino groups.

Swelling curve

The uptake of water by the collagen at different pH values before and after treatment with alkali

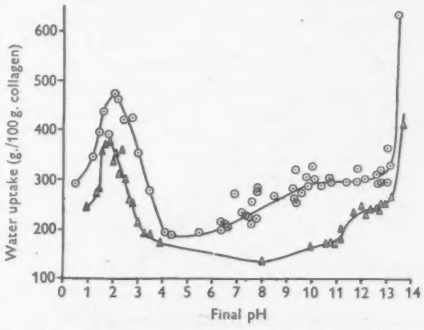

Fig. 2. The water uptake of ox-hide collagen before (triangles) and after (circles) treatment with alkali.

is shown in Fig. 2. After exposure to the acid or alkaline solutions, the pieces of alkali-treated collagen had a roughened appearance especially at high or low pH values. This was probably an indication of the beginning of disintegration. At all pH values the water uptake is appreciably increased by the alkaline treatment, and on the alkaline side of the isoelectric point the shape of the curve is appreciably different from that of the original collagen.

Analysis of samples of skin collagen after alkaline processing ('liming')

The total N, amino N and amide N were determined in a number of skins which had been treated commercially with calcium hydroxide and sodium sulphide to remove the hair ('limed'). The titration curves and arginine contents of three samples were also determined.

Table 4 shows that, with the exception of the sheep-skin collagens, the total N of all the samples was of the same order as that of the alkali-treated ox-hide collagen. The amide-N values varied from 0·25 to 0·47 mmol./g., the sheep skins on the whole having the lowest values. These skins had been treated with a commercial preparation of trypsin ('bating') and with sulphuric acid (approx. 1%) and sodium chloride ('pickling'), and this is probably the cause of the lower values. In a number of cases, the amino N of the commercially treated samples is lower than that of the ox-hide collagen.

The titration curves of sheep skins 1, 2, 3 differ from one another and from the curves for ox-hide collagen, especially in the pH range 6–9.

The curves are more difficult to interpret in terms of the reactive groups than those of the ox-hide collagen. The various sections of the curve, especially those due to titration of unionized carboxyl, imidazole, α-amino and imino groups tend to merge into one another. Treating the curves similarly to that of the alkali-treated collagen, the analyses given in Table 5 were deduced.

Table 4. *Total* N, *amide* N *and amino* N *of collagen of some commercially treated skins*

Source of collagen	Total N (%)	Amide N (%)	Amide N (mmol./g.)	Amino N (%)	Amino N (mmol./g.)
Ox-hide collagen (no alkaline treatment)	18·6	0·66	0·47	0·46	0·33
Sheep skins 1	17·3	0·42	0·30	—	—
2	17·1	0·42	0·30	0·59	0·42
3	17·2	0·35	0·25	0·50	0·36
4	17·4	0·50	0·36	0·45	0·32
5	17·3	0·49	0·35	0·49	0·35
Calf skins 1	18·2	0·56	0·40	—	—
2	18·2	0·52	0·37	—	—
3	18·0	0·55	0·39	0·42	0·30
Ox hide 1	18·1	0·66	0·47	0·46	0·33
2	18·1	0·42	0·30	0·45	0·32
3	18·3	0·46	0·33	0·48	0·34
4	18·1	0·57	0·41	0·42	0·30

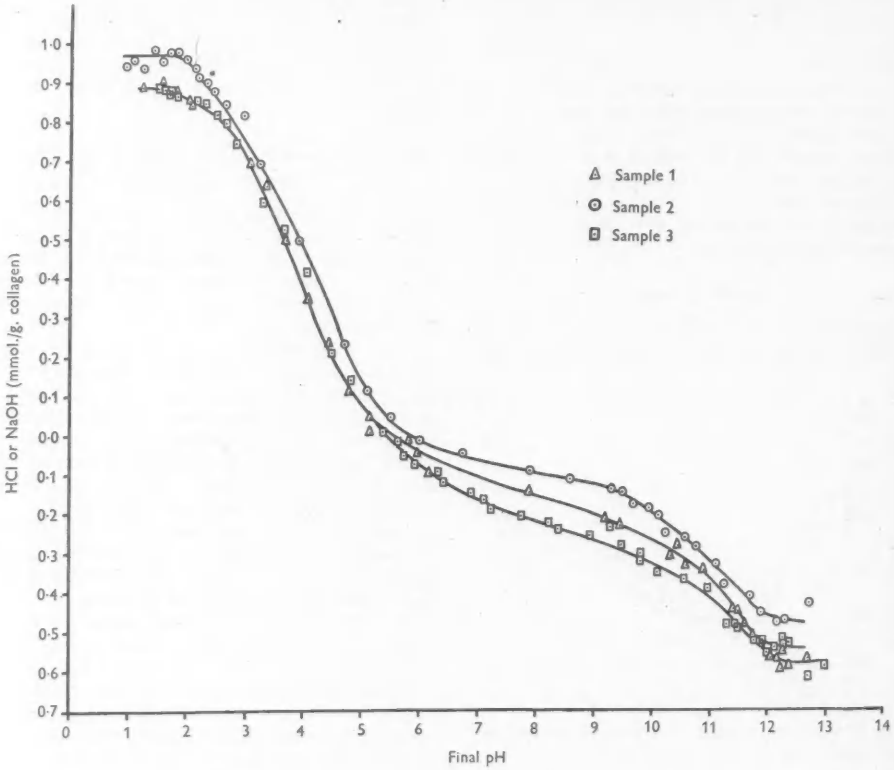

Fig. 3. Titration curves of commercially treated sheep skins.

Table 5. *Analyses of titration curves of alkali-treated sheep-skin collagens*

(Results expressed as mmol./g.)

Group	Method of calculation	Alkali-treated sheep-skin collagen		
		Sample 1	Sample 2	Sample 3
(*a*) Charged carboxyl	Titration from pH 1·5 to isoelectric point	0·89	0·97	0·88
(*b*) Uncharged carboxyl + imidazole	Titration from isoelectric point to pH 7	0·10	0·06	0·16
(*c*) Imidazole, α-amino + imino	Titration from pH 7 to 9·6	0·14	0·10	0·13
(*d*) ε-Amino	Titration from pH 9·6 to 12·5	0·34	0·32	0·26
(*e*) Uncharged carboxyl	(*b*) − 0·03 mmol. imidazole groups titrating in this range	0·07	0·03	0·13
(*f*) Total carboxyl	(*a*) + (*e*)	0·96	1·00	1·01
(*g*) α-Amino + imino	(*c*) − 0·02 mmol. imidazole groups titrating in this range	0·12	0·08	0·11
(*h*) Guanidino	(*a*) + (*e*) − (*b*) − (*c*) − (*d*)	0·38	0·52	0·46
(*i*) Amide	From analysis	0·30	0·30	0·25
(*j*) Dicarboxylic acids	(*f*) + (*i*) − (*g*)*	1·14	1·22	1·15

* It is assumed that terminal carboxyl groups are equivalent to terminal α-amino and imino groups.

DISCUSSION

Considering the comparative severity of the alkaline treatment, the chemical modification of the collagen is comparatively small. Apart from the solubilization of about 5 % of the collagen, the only reaction taking place to any appreciable extent is the hydrolysis of amide groups. Since the loss of amide groups increases with time, it appears probable that all the amide N would be liberated if the treatment were sufficiently prolonged.

The small decrease in arginine content and the presence of urea in solution indicates a breakdown to ornithine, as postulated by Hellermann & Stock (1938) and Warner (1942*a*). A positive test for citrulline in the alkali-treated collagen indicates that the conversion of arginine to citrulline and ammonia also takes place to a very small extent (see Warner, 1942*b*). The amount of ammonia arising from arginine is negligible compared with that due to deamidation.

Nicolet, Shinn & Saidel (1942) have shown that treatment of silk fibres with boiling 0·1 M-alkali for 1–2 hr. causes partial destruction of serine and threonine, producing an approximately equivalent number of amide groups. There is no evidence that such a reaction occurs even to a limited extent when collagen is treated with alkali in the cold for periods up to 14 days.

The ornithine produced by modification of the guanidino groups could account for the small increase in the base bound between pH 9·6 and 12·5, and for the greater part of the increase in free amino N. On this basis, there can be little, if any, hydrolysis of peptide links involving amino groups in the alkali-treated collagen, and the increase in acid-binding capacity (0·06 mmol./g.) must presumably be due to release of basic groups other than amino groups. It is possible that these are imino groups, derived from hydrolysis of peptide links involving proline or hydroxyproline. Whilst this would suggest that such peptide links are more alkali-labile than those involving amino groups, Gordon, Martin & Synge (1943), from a study of the partial acid hydrolysate of gelatin, inferred that they were resistant to acid hydrolysis at 37°.

Although there is only a small increase in the number of terminal groups in the alkali-treated collagen, an appreciable amount (5 %) of the collagen was rendered soluble by the treatment. Some of this was presumably in the form of large polypeptides, since it was precipitated on neutralization of the solution; peptides which did not precipitate probably account for nitrogen in the solution in excess of that due to ammonia and urea. The amino-N figure for the solution suggests that this fraction must be in the form of amino-acids or small peptides. Evidence deduced from the titration curve suggests that this soluble fraction is rich in the dicarboxylic acids. Martin (1946) has recently demonstrated the presence of aspartylglutamic, glutamylglutamic, and glutamylaspartic acids in the partial hydrolysate of wool, and if these two dicarboxylic acids also occur together in collagen, breakdown could readily lead to their specific loss.

Consideration of the swelling curves indicates that physical as well as chemical changes take place during the alkaline treatment, for the changes in the curve can only be partially accounted for by changes in the reactive groups. The water uptake in the isoelectric range is increased, the increase in water uptake in acid solutions is greater than corresponds to the increase in basic groups, and the increase between pH 7 and 9 can only partially be accounted for by the titration of uncharged carboxyl groups liberated by hydrolysis of amide groups. The cause of the shift in the pH at which water uptake in alkaline solutions reaches a maximum from 12·5 to 9·5 is not clear.

Examination of the commercially treated skins indicates that similar changes have taken place; the total N and amide N of these were lower than those of the original ox-hide collagen, but the amino N was in some cases lower, instead of higher as might have been expected from the laboratory experiment. It is possible that this may be due to bacterial deamination at some stage in the processing of the skins (Stuart, 1946). The total N of the sheep skins was particularly low, and is not accounted for by the lower values of the amide N and arginine N as compared with the original ox-hide collagen, which suggests the possibility that the composition of sheep-skin collagen may differ from that of ox hides.

SUMMARY

1. Hydrolysis of amide groups is the main reaction taking place when collagen is treated with alkali at pH 13·0. A small number of the guanidino groups is converted to ornithine and urea, and a still smaller fraction to citrulline and ammonia. There is a small increase in the total number of basic groups and in the number of free amino groups; it is suggested that some peptide links involving proline and hydroxyproline are broken. The hydroxyamino-acids, serine, threonine and hydroxylysine, are unaffected by the treatments. Approximately 5 % of the collagen is solubilized.

2. Alkali-treated collagen swells more than the original collagen.

3. Skins treated commercially with alkaline solutions undergo changes similar to those found in laboratory experiments.

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The Nutrition of the Larva of *Aëdes aegypti* Linnaeus

3. LIPID REQUIREMENTS

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In a previous communication (Golberg, De Meillon & Lavoipierre, 1945) we showed that water-extracted yeast residue, supplemented by a number of water-soluble vitamins, constitutes an adequate medium for the growth and metamorphosis of the larva of *Aëdes aegypti* Linnaeus. From this starting point our investigations were concerned with the part played by lipids in the nutrition of the mosquito larva.

Considering the ability of insects to transform carbohydrate and protein into fat (evidence summarized by Wigglesworth, 1942), it is surprising to find that many insects are incapable of synthesizing sterols; or at least of producing them in sufficient quantities to cover their needs for growth and particularly for metamorphosis. The need for preformed sterol in the diet of many insects has been conclusively demonstrated. Yet serious consideration does not appear to have been given to the possibility that non-steroid lipids may be capable of fulfilling the same nutritive purpose. For example, lecithin has been employed as a component of diets for *Drosophila* (Bacot & Harden, 1922; Guyénot, 1917; Lafon, 1937), and frequent reference is made in the literature to essential lipid factors present in wheat, wheat germ, cod-liver oil, butter fat, and ethanolic extract of yeast. In two instances, in which attempts have been made to fractionate the fat and isolate the active agent, the conclusion was reached that cholesterol was the essential constituent (Hobson, 1935; van't Hoog, 1936). Fraenkel & Blewett (1945) found that, in addition to cholesterol, insects of the genus *Ephestia* require linoleic acid and vitamin E.

Gay (1938) and subsequent workers (Fraenkel, Reid & Blewett, 1941; Fraenkel & Blewett, 1943) employed yeast in the growth media used for studying the sterol requirements of insects. Although the proportion of yeast in the dietary solids varied from 5 up to 96%, they, nevertheless, found it necessary to add a further quantity of cholesterol to the diet, the minimum being 1 mg. sterol/3 g. diet (Fraenkel *et al.* 1941). Dried yeast has a fat content of 7% (Newman & Anderson, 1933) of which approximately one third consists of sterols (Smedley-MacLean & Thomas, 1920). It would appear that this level of sterols is inadequate for insects normally feeding on flour or other stored food, particularly when the yeast constitutes only 5% of the artificial diet. In the case of *Aëdes* larvae, however, when the whole of the solid portion of the growth medium was composed of yeast, no added sterols or other lipids were needed.

EXPERIMENTAL

Aëdes aegypti *L.* The strain of mosquitoes used was the same as in our previous investigations (De Meillon, Golberg & Lavoipierre, 1945; Golberg *et al.* 1945).

Method of obtaining sterile eggs and larvae of Aëdes aegypti. In order to ensure that the eggs used were not more than 24 hr. old, the following procedure was employed: gravid females from the insectary were confined, singly, in sterile test tubes, together with 2–3 ml. sterile distilled water and incubated at 28°. The tubes were examined every morning and the females liberated from those tubes containing eggs. The eggs and water were poured with aseptic precautions into a fresh sterile tube, and the water withdrawn.

Tubes with stranded eggs were now half-filled with White's solution (White, 1931), left for 30 min., the fluid removed and the eggs washed three times with sterile distilled water. In the final washing a platinum loop was used to dislodge any eggs adhering to the sides of the tube. The sterilized eggs were transferred directly to tubes containing an autoclaved solution of glucose (1%) and Tatum's (1939) salt mixture (0·15%), previously adjusted to pH 6·5. Incubation at 28° was continued for 96 hr. after the larvae had hatched, and served to detect contaminated batches. Those found to be sterile were used forthwith.

Preparation of media. As a rule each experimental medium consisted of a liquid and a solid component, prepared and sterilized separately as follows: *The liquid component* was invariably prepared fresh and sterilized on the same day by pressure filtration through a pad. The sterile filtrate was collected in a special apparatus designed to facilitate rapid distribution of the liquid into tubes containing the sterile solid fraction. In general 10 ml. were measured into each tube. Where sodium caseinate was used, it was autoclaved and added separately. *The solid component* was sterilized by autoclaving for 30 min. at 15 lb. pressure. A quantity of 20–50 mg. per tube was adequate, but observation of the larva was facilitated by adding an amount sufficient to fill the rounded portion of the tube. Lipids were introduced into the solid fraction of the medium by solution in a small volume of pure ether (or other suitable volatile solvent) and stirring with powdered cellulose or kaolin until the solvent had evaporated. Such mixtures were autoclaved in the dry state.

Media were tested for sterility in the usual manner.

Observations of development and methods of recording them. The stage of development reached by each larva was recorded every morning and evening, and the time taken for the change from one stage to the next was calculated to the nearest half-day. In considering the results of experiments, it was thus possible to compare the times taken for each stage ('growth rates') and also the number of larvae reaching that stage ('survival rates'). It is realized that the growth rates recorded are subject to a maximum error of +0·5 day, but this does not affect the standard deviations or the significance of differences between groups.

In most tables the results have been condensed, but all the data are available in the same detail as in Tables 1 and 2.

Materials and composition of media

The basal medium (*LM*) contained glucose (10 g./l.), salt mixture (Tatum, 1939) (1·5 g./l.) and the following vitamins (in mg./l.): aneurin chloride, 10; riboflavin, 20; pyridoxin hydrochloride, 10; calcium D-pantothenate, 10; nicotinic acid, 10. The pH was adjusted to 6·3–6·5.

For use with sodium caseinate the basal medium was prepared in double strength and 5 ml. filtered medium added to 5 ml. autoclaved sodium caseinate.

Sodium caseinate was at first prepared from purified casein. Later, S.M.A. Co. 'Vitamin test' casein was used, and the pH adjusted to 6·6. Immediate autoclaving at 18 lb. for 45 min. was essential to prevent contamination. Analysis showed the average concentration of sodium caseinate in the final medium to be 0·95% (w/v).

Yeast autolysate. Difco 'Bacto yeast extract' was used in a concentration of 2% (w/v).

Yeast residues were prepared from fresh brewer's yeast which was dehydrated with ethanol, extracted at room temperature for 24 hr. with ethanol, followed by a mixture of ethanol and light petroleum (1 : 1). After five additional extractions with boiling mixtures of ethanol and benzene (2 : 1) (Bills, Massengale & Prickett, 1930) it was dried in air, powdered and sieved.

Newman & Anderson (1933) showed that treatment with ethanolic hydrogen chloride was necessary for the extraction of a small quantity of yeast fat which cannot be removed by neutral solvents. Accordingly, the yeast residue as prepared above was incubated for 2 days at 37° with 1% (w/w) ethanolic hydrogen chloride, after which it was washed and dried as before.

Yeast or yeast residue was freed from water-soluble constituents by repeated extraction with boiling water and finally by continuous extraction with boiling water in a Soxhlet apparatus for 48 hr.

Lipids. Freshly prepared and purified egg-yolk lecithin and beef-brain cephalin were submitted to additional purification as described by Weil-Malherbe & Dickens (1944). Careful testing failed to reveal the presence of sterols in the purified materials. Sphingomyelin (beef brain) was purified by the procedure of Levene (1916). Kerasin was prepared from the fresh spleen of a case of Gaucher's disease, supplied by Dr J. A. Murray of this Institute.

Steroids. Many of the sterols and derivatives used were prepared by standard procedures. The remainder, including sex hormones and related compounds, were obtained from Drs F. W. Fox and J. Gillman, Prof. I. M. Heilbron and Dr E. R. H. Jones, Mr F. A. Robinson (Glaxo Laboratories Ltd.), Profs. L. Ruzicka and Pl. A. Plattner, Dr E. Schwenk (Research Division, Schering Corporation) and Dr H. A. Shapiro.

All steroids used were checked for purity and where necessary submitted to further purification. Only in the case of 7-dehydrocholesterol was the test material of doubtful purity.

Methyl linoleate and linolenate were obtained from Prof. T. P. Hilditch.

Folic acid. A concentrate of the ammonium salt of folic acid, of potency 5000, obtained from Prof. R. J. Williams, was used in a concentration of 20 mg./l. of medium.

Pteroylglutamic acid was made available to us by Dr E. L. R. Stokstad (Lederle Laboratories). It was used in a concentration of 25 mg./l.

RESULTS

The growth-promoting properties of fat-free yeast and yeast lipids

The basal medium (*LM*) containing glucose, salts, aneurin, riboflavin, pyridoxin, nicotinic and pantothenic acids, when combined with fresh brewer's yeast, produced excellent growth of the larvae. Gradual removal of the fats from the yeast decreased the rate of growth and the number of pupae and adult mosquitoes. Finally, after extraction of the yeast residue with ethanolic hydrogen chloride, there was complete failure to emerge from the pupal stage (Table 1). Recombination of the final yeast residue with the yeast lipids led to normal growth and survival rates, provided that the lipid concentration did not exceed 4%. At 8% lipid concentration the medium was very toxic.

Further experiments were designed to supplement fat-free yeast residue with yeast lipid fractions and with a variety of lipids from other sources. The crude yeast fat was able to produce normal growth. Its ether-soluble fraction did the same. Of the other fractions tested, that obtained by steam-distilling yeast fat was remarkably toxic. In a concentration of 0·15% on the solid component of the medium it completely prevented growth of the larvae. At higher concentrations it killed them within 24 hr.

Admixture of fat-free yeast residue with cholesterol, purified ovolecithin or beef-brain kephalin in each case resulted in growth to the adult stage. The best results were obtained by the use of all three lipids in a total concentration of 2·5%, resulting in growth and survival rates closely similar to those observed with yeast fat.

Table 1. *Rates of growth and survival of mosquito larvae on media containing fat-free yeast*

(Here and in subsequent tables: No. = number in stage of development indicated. The number of first-instar larvae taken was approximately 20 per experiment. Mean; S.D. = mean and standard deviation of time (in days) taken to change from previous stage of development.)

	Larval instars								Adults		
	II		III		IV		Pupae		Total time taken		
Medium	No.	Mean; S.D.	No.	Mean; S.D.	No.	Mean; S.D.	No.	Mean; S.D.	No.	Range	Mean; S.D.
LM, with additions as indicated:											
Fresh brewer's yeast	63	1·9±0·36	63	1·1±0·19	63	1·1±0·29	60	2·6±0·50	57	7·5–11·5	8·8±0·60
Fat-free yeast residue	48	2·6±0·48	48	2·1±0·57	48	3·2±1·03	17	10·7±2·36	0	—	—
Fat-free yeast + yeast lipids (2·5%)	23	2·2±0·29	22	1·3±0·41	22	1·6±0·38	20	3·0±0·62	18	8·5– 9·5	9·1±0·49
Water-extracted fat-free yeast	7	4·1±1·60	7	2·9±0·83	0	—	—	—	—	—	—
Water-extracted fat-free yeast + folic acid	18	1·9±0·99	16	1·9±0·75	1	3·0	—	—	—	—	—
Fat-free water-extracted yeast	22	3·8±0·86	20	5·0±1·65	11	5·4±1·09	0	—	—	—	—
Fat-free water-extracted yeast + pteroylglutamic acid	21	1·9±0·63	21	1·6±0·84	12	3·8±3·61	2	5·8	0	—	—
Fat-free water-extracted yeast + yeast autolysate	17	5·0±3·08	16	1·4±0·45	14	3·2±1·96	1	15·0	0	—	—
Fat-free yeast + cholesterol	20	2·7±0·92	20	1·4±0·54	20	1·7±0·55	17	3·9±0·62	16	9·5–15·5	11·6±1·60
Fat-free yeast + lecithin	19	2·8±0·88	19	1·9±0·93	17	2·5±1·32	16	3·8±0·50	16	9·5–16·5	12·6±2·10
Fat-free yeast + kephalin	22	2·9±0·98	20	2·3±0·67	20	2·8±1·48	14	5·5±1·44	12	11·5–20	15·1±2·73
Fat-free yeast + cholesterol, lecithin and kephalin (1 : 1 : 1)	19	1·6±0·16	19	1·2±0·31	19	1·3±0·49	19	3·4±0·55	19	8·5–10·5	9·7±0·85

Exhaustive extraction of fat-free yeast with water rendered it incapable of supporting growth, even to the fourth larval instar. The addition of folic acid to the medium made no essential difference. Reversal of the order of extraction, using water first and fat solvents later, yielded a residue which promoted better growth and afforded clear evidence of the effect of lipid deficiency on growth and survival of mosquito larvae (Table 1).

Growth on other fat-free media

A study was made of the growth of larvae on fat-free media not containing solid yeast. Casein, as the source of protein, gave best results when present in solution as the sodium salt. By incorporating yeast autolysate in the medium it was found that the fat-free yeast residue could be dispensed with (Table 2). In none of our experimental media could a need for

solid material be demonstrated. However, it was convenient to use a solid in finely divided form as a means of introducing lipids into the medium; and for this purpose cellulose, cellulose acetate or kaolin were equally effective.

Fatty acids and triglycerides

Since a number of compounds seemed capable of bringing about the lipid effect, it was deemed advisable to make sure that this property did not extend to the common fatty acids and triglycerides. None of the following could replace cholesterol: palmitic, stearic and oleic acids; tripalmitin, tristearin and triolein. Lauric and myristic acids were highly toxic; so too was oleic acid, even in a concentration of 0·5 % on kaolin. More highly unsaturated fatty acids—linoleic and linolenic acids, even when used with α-tocopherol (Fraenkel & Blewett, 1945) were inactive. Furthermore, all the above-mentioned compounds proved incapable of supplementing cholesterol in the way in which lecithin and kephalin had done. Similarly, esterification of cholesterol with acetic, oleic, palmitic, stearic, cerotic and arachidic acids had no striking beneficial effect on larval growth and survival (Table 3).

Table 2. *Rate of growth and survival of mosquito larvae on media not containing fat-free yeast residue**

	Larval instars								Adults		
	II		III		IV		Pupae		Total time taken		
Medium	No.	Mean; S.D.	No.	Mean; S.D.	No.	Mean; S.D.	No.	Mean; S.D.	No.	Range	Mean; S.D.
LM + yeast autolysate, with additions as indicated:											
Casein	36	2·7±1·41	35	2·2±0·78	16	5·1±4·37	0	—	—	—	—
Sodium caseinate	20	3·1±0·33	20	1·4±0·39	18	2·4±1·42	8	5·9±1·19	0	—	—
Sodium caseinate + cellulose	21	1·9±0·37	20	1·6±0·21	16	3·0±1·44	3	7·0	1	—	12·5
Sodium caseinate + kaolin	34	1·8±0·63	34	1·9±0·48	25	3·2±1·85	7	6·5±0·76	3	12·5–15	14·0
LM + yeast autolysate + sodium caseinate + lipids in 2·5 % concentration on cellulose:											
Cholesterol	17	2·0±0·51	17	1·3±0·24	17	1·8±0·25	13	3·8±0·75	9	9·5–12·5	11·0±1·04
Lecithin	24	1·8±0·38	24	1·4±0·36	24	1·9±0·39	22	4·4±1·14	16	9·5–14·5	11·9±1·49
Kephalin	25	1·7±0·32	25	1·6±0·42	24	2·4±1·36	8	5·3±2·10	1	—	10·0
Cholesterol + lecithin + kephalin (1 : 2 : 1)	40	2·1±0·67	40	1·5±0·42	39	1·9±0·51	34	3·4±0·65	32	9·5–13	10·8±1·09
Sphingomyelin	17	2·4±0·78	17	1·2±0·60	17	1·7±0·69	11	3·9±0·63	7	9·5–11	10·5±0·60
Kerasin	22	2·3±0·89	22	1·3±0·42	22	1·8±0·38	18	3·8±0·65	16	10·5–13	11·4±0·92
β-Carotene	44	3·3±0·76	44	2·0±0·60	44	2·8±1·48	31	6·2±2·10	23	11 –29	16·9±2·81
α-Tocopherol	37	2·3±0·39	37	2·2±0·61	30	3·4±0·81	15	8·3±2·48	11	13 –23·5	16·8±2·11
α-Tocopheryl acetate	15	2·4±0·60	15	2·0±0·45	15	2·7±0·69	12	8·4±2·63	8	12·5–24·5	17·5±3·08

* For explanation of headings see Table 1.

Table 3. *The effect of cholesteryl esters on growth and metamorphosis of mosquito larvae*

(Medium: *LM* + sodium caseinate + yeast autolysate + cholesteryl ester (2·5 %) on cellulose.)

Cholesteryl ester tested	No. of second larval instars	No. of adult mosquitoes	Total time taken	
			Mean	S.D.
Unesterified	22	11	11·8	±0·97
Acetate	23	14	10·6	±0·69
Palmitate	17	12	13·5	±1·74
Stearate	20	11	13·4	±2·92
Oleate	14	5	13·3	±1·29
Cerotate	18	6	13·7	±1·32
Arachidate	17	9	16·9	±4·36
p-Aminobenzoate	17	15	18·0	±1·90
Nicotinate	19	15	12·4	±2·37

A medium was thus available for testing the effects of various lipids. Cholesterol and lecithin were capable of bringing about pupation of the larvae and emergence of adults, but cephalin was totally ineffective. On combining these lipids in pairs, or using all three in various proportions, the rates of growth were but little affected, while the rates of survival varied considerably. The highest proportion of adult mosquitoes (80 %) resulted from the use of lecithin, cholesterol and kephalin in the proportion of 2 : 1 : 1. This mixture was adopted as the standard source of lipids for subsequent 'synthetic' growth media.

Of the other lipids tested under the same conditions, only kerasin and, to a lesser extent, sphingomyelin, β-carotene and α-tocopheryl acetate, were capable of replacing cholesterol.

Lipids in solution

On testing the effect of presenting the lipids in solution, it was found that neither lecithin nor cholesterol had any apparent effect. Nor was lecithin in solution able to improve the larval response to cholesterol present in the solid state (Table 4). Fatty acids in solution as the sodium salts were tested under the same conditions: palmitate and stearate had no effect and oleate displayed no toxicity. The concentrations tested were of necessity rather low (0·25 %).

Table 4. *The effect of lipids in solution on growth and metamorphosis of mosquito larvae*

(Medium: *LM* + sodium caseinate + yeast autolysate.)

Lipid in solution	Concentration (g./l.)	Lipid on cellulose (4 % concentration)	No. of second larval instars	No. of adult mosquitoes	Total time taken Mean	S.D.
None	—	None	28	2	13·0	
Lecithin	0·4	None	69	5	14·5	
Lecithin	0·25	None	15	2	13·0	
Lecithin	0·4	Cholesterol	23	10	10·9	±0·87
Lecithin	0·25	Cholesterol + kephalin (1 : 1)	63	38	12·5	±0·99
Lecithin	0·4	Kephalin	18	1	10·5	
None	—	Lecithin	24	16	11·9	±1·49
Cholesterol	0·05	None	23	1	18·5	

Table 5. *The effects of various sterols on growth and metamorphosis of mosquito larvae*

(Medium: *LM* + sodium caseinate + yeast autolysate + sterol (2·5 %) on cellulose.)

Sterol tested	No. of second instar larvae	No. of adult mosquitoes	Total time taken Mean	S.D.
Cholesterol	22	11	11·8	±0·97
Cholesterol (purified through the dibromide)	39	15	11·5	±1·53
Coprosterol	22	10	13·5	±1·87
β-Sitosterol	43	32	10·7	±1·12
Stigmasterol (acetate)	23	10	10·6	±0·59
Ergosterol	21	7	9·2	±0·84
Zymosterol	21	1	10·0	

Table 6. *The ability of various sterols to replace cholesterol in a medium containing fat-free water-extracted yeast residue*

(Medium: *LM* + sodium caseinate + yeast autolysate + sterol (2·5 %) on fat-free water-extracted yeast.)

Sterol tested	No. of second instar larvae	No. of adult mosquitoes	Total time taken Mean	S.D.
Cholesterol	20	19	9·4	±0·96
Coprosterol	19	19	12·5	±1·80
β-Sitosterol	15	14	9·5	±1·30
Stigmasterol (acetate)	20	20	8·8	±0·73
Ergosterol	18	12	9·4	±0·84
Zymosterol	19	16	14·1	±2·56
None	17	0	—	

The ability of other sterols to replace cholesterol in the larval diet

It is most unlikely that cholesterol is normally available to the mosquito larva in its natural habitat. Hence the question arises: to what extent can other sterols replace cholesterol in the larval diet? The results given in Table 5 indicate considerable variation in this respect. In fact, the principal sterols of yeast, when provided in pure form, appear incapable of utilization by the mosquito larva. However, under the conditions of these experiments no sterol—not even cholesterol—produced a wholly satisfactory response, particularly with regard to the proportion of larvae reaching the adult stage. In order to render the medium more fully adequate for the promotion of optimal growth and survival, the powdered cellulose was replaced by a water-extracted yeast residue from which all lipids soluble in neutral organic solvents had been removed. This preparation still contained a small amount of lipid extractable by incubation with ethanolic hydrogen chloride. The residual lipid did not prevent a complete failure to pupate in the absence of added sterol; yet it ensured that when cholesterol was added excellent growth and survival resulted (Table 6). Under these

conditions the plant sterols were fully equal to cholesterol in their effects. The mycosterols were definitely inferior to the others, zymosterol producing a poor rate of growth, ergosterol a low proportion of adults.

Modifications of sterol structure

These results made it desirable to inquire into the effects of modifications in the sterol structure, and to investigate the relationship of the activity of such sterol derivatives to other dietary factors. For this purpose each compound was tested on three solid components in the usual liquid medium. The solids used were: cellulose, fat-free water-extracted yeast, and the yeast residue prior to extraction with ethanolic hydrogen chloride. The conclusions to be drawn from the results, some of which are given in Table 7, may be summarized thus: (*a*) none of the compounds tested showed any striking degree of activity on cellulose; most were completely inactive; (*b*) in the presence of both types of yeast residue the following compounds displayed activity: β-cholestanol, cholesterol dibromide, Δ^4-3(β):6-dihydroxycholestene, 3(β)-hydroxy-6-ketocholestane. There was remarkable correspondence between the growth and survival rates on the two types of yeast residue; (*c*) all other compounds tested proved inactive. These included the following cholesterol derivatives: Δ^4-cholestenone* and its enol acetate*, α-7-hydroxy- and 7-ketocholesterols, $\Delta^{4,6}$-3-ketocholestadiene*, $\Delta^{3,5}$-7-ketocholestadiene* 3(β):5:6-(*trans*)trihydroxycholestane*, *cis*- and *trans*-Δ^5-cholestene-3:4-diols and their diacetates, and methyl Δ^5-3-acetoxybisnorcholenate. Calciferol was inactive, as well as the following steroid hormones and related compounds of the pregnane, androstane and oestrane series: Δ^5-pregnen-3-ol-20-one; dehydro*iso*androsterone acetate; progesterone; deoxycorticosterone acetate; 17-ethinyl- and 17-methyl-testosterone; testosterone itself and its propionate; Δ^4-androstene-3:17-dione; pregnane-3(α):20α-diol; *allo*pregnane-3(α):20α-diol; androsterone; *allo*pregnane-3:20-dione; oestrone and oestradiol benzoate; (*d*) among the inactive compounds were some which were markedly growth-inhibitory (marked with an asterisk above).

Table 7. *The growth-promoting properties of sterol derivatives*

(Media: *LM* + sodium caseinate + yeast autolysate + test compound (2·5%) on: *A*, cellulose; *B*, yeast extracted with neutral organic solvents and water; *C*, yeast *B* further extracted with ethanolic hydrogen chloride.)

Sterol derivatives tested	No. of second instar larvae	No. of fourth instar larvae	No. of adult mosquitoes	Total time taken Mean	S.D.
β-Cholestanol on *A*	22	14	6	12·4	±0·61
B	18	17	12	12·8	±1·04
C	17	17	12	12·5	±1·77
Cholesterol dibromide on *A*	19	19	9	11·7	±1·05
B	22	22	21	13·0	±2·86
C	20	19	19	12·3	±1·31
3(β)-Hydroxy-6-ketocholestane on *A*	16	12	0	—	
B	18	17	9	13·6	±2·07
C	18	16	7	14·9	±1·82
Δ^4-3(β):6-Dihydroxycholestene on *A*	21	13	0	—	
B	22	22	20	12·1	±1·58
C	19	17	14	12·8	±1·93
7-Dehydrocholesterol on *A*	21	18	1	12·0	
B	17	17	14	12·5	±1·60
C	18	18	17	11·8	±1·27

DISCUSSION

In the experiments described above, the progress of each individual larva was recorded. In consequence it was possible to demonstrate that a lipid-free medium supports larval growth, albeit at a somewhat reduced rate, to the fourth instar. The effect of lipid deficiency reveals itself most frequently in a failure to undergo metamorphosis. In those few instances in which pupation does occur, it takes place after a considerable delay, and only a small proportion of adults emerge.

Lipid deficiency can be corrected, but not completely, by cholesterol or lecithin, kerasin and, to a lesser degree, by other lipids. In addition, the action of lipids can be supplementary, and a mixture of cholesterol, lecithin and kephalin gives better results than either cholesterol or lecithin alone (kephalin alone is inactive). There are thus two aspects of lipid action to be considered, which may be referred to briefly as replacement and supplementation of cholesterol.

The ability of lecithin to replace cholesterol cannot be attributed to the presence in it of sterol as an impurity (see p. 373). Also the activity of lecithin

does not appear to be due to component unsaturated fatty acids, since such fatty acids proved incapable of replacing or supplementing cholesterol. This, taken in conjunction with the fact that relatively few steroid derivatives evince this type of activity, leads to the conclusion that the specialized nutritive power is a function of special types of molecules. It is impossible to state at this stage what these molecules have in common. Theirs is not a physicochemical influence on the external medium of the mosquito larva since, when present in solution in the medium, they are inactive. On the other hand, there is a definite parallelism between their effects on the mosquito larva and their ability to overcome the growth inhibition of Gram-positive bacteria induced by linoleic acid. This effect Kodicek & Worden (1945) have attributed to surface activity.

Supplementation of cholesterol is brought about most effectively by fat-free yeast residue. However, attempts to isolate a specific supplementary factor from the yeast residue have failed. One is reminded of the animal dietary factor found necessary by Dorée & Gardner (1938) and Rosenheim & Webster (1941) for the conversion of cholesterol to coprosterol and for analogous metabolic changes. It is suggestive too that some cholesterol derivatives which are inactive when tested alone evince a measure of activity in the presence of the yeast factor.

While some sterols are capable of replacing cholesterol in the diet of the mosquito larva, on the whole little modification of sterol structure is tolerated. This statement is borne out by the findings of other workers who have studied the ability of insects to utilize sterols and cholesterol derivatives (Fraenkel *et al.* 1941; Fraenkel & Blewett, 1943; van't Hoog, 1936). The paucity of the information available makes it all the more regrettable that Fraenkel & Blewett (1943) used impure cholesterol derivatives in their investigations. The most exhaustive study of this type was made by Cailleau (1937) on the ability of sterols to act as growth factors for *Trichomonas columbae*. Although her results are not necessarily applicable to insects, it is interesting that in most instances the ability of the flagellate to utilize sterols, and particularly cholesterol derivatives, runs strictly parallel to that of the mosquito larva. The outstanding differences lie in coprosterol and stigmasterol, which Cailleau found to be inactive.

Finally, it had been hoped by means of these studies to throw some light on the possible metabolism of cholesterol in the mosquito larva. Δ^4-3:6-Dihydroxycholestene and 3-hydroxy-6-ketocholestane are the only two cholesterol transformation products which are active for the mosquito larva, and at the same time may be regarded as likely to occur in normal cells during life (Haslewood, 1944). Even these are active only in the presence of the yeast factor. The conversion of cholesterol to coprosterol takes place through the intermediate stage of Δ^4-cholestenone (Schoenheimer, Rittenberg & Graff, 1935; Anchel & Schoenheimer, 1938; Marker, Wittbecker, Wagner & Turner, 1942). Turfitt (1944, 1946) demonstrated the microbiological conversion of cholesterol to Δ^4-cholestenone. In the mosquito larva Δ^4-cholestenone is not only inactive but also strongly inhibitory; conversion to the enol acetate does not abolish the inhibitory effect. In keeping with these observations is the lack of activity of *cis*-Δ^5-cholestene-3:4-diol, which Rosenheim & Starling (1937) found to be readily dehydrated to cholestenone by mild acid treatment.

SUMMARY

1. A study has been made of the part played by lipids in the nutrition of the mosquito larva. The lipids were mixed intimately with an inert solid such as cellulose or kaolin or with fat-free yeast residue and introduced into a basal liquid medium.

2. It was found that in a fat-free medium the rates of growth and survival of the mosquito larva do not fall far short of normal. There are, however, few pupae and very few adult mosquitoes.

3. The larval need for lipid may be supplied by cholesterol or ovolecithin and, less successfully, by a number of other lipids. Of all those tested, no single compound, and no cholesterol ester, was capable of producing normal growth and survival unless fat-free yeast residue was included in the growth medium. Mixtures of lipids were more effective.

4. The following compounds proved inactive: saturated and unsaturated fatty acids; triglycerides; lecithin and cholesterol *in solution* in the growth medium.

5. A number of steroids, including cholesterol derivatives, were tested in the presence or absence of fat-free yeast residue. All but four were inactive.

6. The need for special lipids appears to be common to many insects and lower organisms and to be capable of being satisfied only by a limited number of compounds. The possibility exists that the effect exerted by these compounds is physicochemical rather than nutritional.

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The Nutrition of the Larva of *Aëdes aegypti* Linnaeus

4. PROTEIN AND AMINO-ACID REQUIREMENTS

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The state of confusion which exists in the literature on the protein and amino-acid requirements of insects may be attributed to two causes: failure to maintain strictly sterile conditions and failure to ensure adequacy of the medium with respect to all factors other than protein. In turning our attention to the mosquito larva's needs for protein and amino-acids we have sought to avoid both pitfalls and can claim to have succeeded with regard to the first. As regards the second, our efforts have not yet culminated in a medium composed of purified individual chemical substances which could produce growth equal to that attained with the original fresh brewer's yeast medium (De Meillon, Golberg & Lavoipierre, 1945). Until that stage is reached, a final answer to some of the questions raised in this paper cannot be given. Meanwhile, it should be emphasized that growth on the media used fell short of what had been produced in the presence of yeast residue. Not only were the growth and survival rates somewhat poorer, but it was obvious that the spontaneity and uniformity which had characterized the response to whole yeast was lacking.

EXPERIMENTAL

Methods. The methods used were the same as in our previous investigations (Golberg & De Meillon, 1947). The microbiological estimation of L-valine was carried out by the method of McMahan & Snell (1944).

Media. The basal medium (*CM*) consisted of two components: (i) *a solid component* made up of 0·25 g. of a lipid mixture (lecithin, cholesterol and kephalin in the proportions 2:1:1) intimately mixed with 10 g. of finely powdered cellulose, and sterilized by autoclaving for 30 min. at 15 lb. pressure; and (ii) *a liquid component* made up of two halves: one half, which was sterilized by filtration through a pad, contained glucose (10 g./l.), yeast autolysate (20 g./l.), Tatum's (1941) salt mixture (1·5 g./l.) and vitamins (aneurin chloride, 10 mg./l.; riboflavin, 20 mg./l.; pyridoxin hydrochloride, 10 mg./l.; calcium D-pantothenate, 10 mg./l.; and nicotinic acid, 10 mg./l.). The other half of the liquid component, which was sterilized by autoclaving for 45 min. at 18 lb. pressure, contained the protein in solution.

Amino-acid mixtures were added to the first half of the liquid component prior to filtration. In all instances sterilization of the medium followed its preparation with the shortest possible interval for it was found that even slight contamination incidental to keeping a protein or amino-acid

Table 1. *Amino-acids of media*

(g./l.)

	Amino-acid mixtures									
	X	*Y*	*B*	*C*	*D*	*I*	*N*	*O*	*P*	*R*
Glycine	—	—	1·4	1·4	1·4	2·0	1·4	2·0	2·0	2·0
DL-Alanine	—	—	0·5	0·6	0·6	0·2	0·6	0·6	—	—
DL-Valine	0·4	0·8	0·2	0·8	0·8	0·8	0·8	0·8	1·0	—
L-Leucine	—	0·8	0·2	0·8	0·8	0·8	0·8	1·0	1·0	1·0
DL-Isoleucine	0·8	0·8	0·4	0·8	0·8	0·8	0·4	0·6	1·0	1·0
DL-Proline	—	—	0·6	0·6	0·6	0·2	—	—	—	—
L-Hydroxyproline	—	—	0·2	0·2	0·2	0·2	—	—	—	—
DL-Phenylalanine	—	0·6	0·2	0·6	0·6	0·6	0·6	0·6	0·6	—
DL-Glutamic acid	—	—	0·3	0·4	—	—	—	—	—	—
DL-Aspartic acid	—	—	0·2	0·4	—	—	—	—	—	—
DL-Serine	—	—	0·2	0·2	0·2	0·2	—	—	—	—
L-Tyrosine	0·4	0·8	0·2	0·8	0·8	0·4	0·8	0·8	0·8	0·8
L-Histidine HCl (hydrate)	—	0·4	0·2	0·4	0·4	0·8	0·4	0·4	0·8	0·8
L-Arginine HCl	—	—	0·5	0·6	0·6	0·8	0·6	0·6	0·8	1·0
L-Lysine HCl	—	0·4	0·4	0·4	0·4	0·8	0·4	0·8	0·8	0·8
L-Tryptophan	0·4	0·4	0·2	0·4	0·4	0·8	0·4	0·6	0·8	0·8
DL-Threonine	0·4	0·6	0·2	0·6	0·6	0·8	0·4	0·6	1·0	1·0
DL-Methionine	—	0·6	0·4	0·6	0·6*	0·8	0·6	0·6	0·6	1·0
L-Cystine	0·4	0·02	0·2	0·02	0·02	0·02	0·02	0·06	0·04	0·02
Total concentration of amino-acids (%)	0·28	0·62	0·67	1·06	0·98	1·10	0·82	1·01	1·12	1·02

* Later increased to 1·0 g./l.

solution might result in extraneous growth-stimulating effects on the larvae.

The compositions of amino-acid mixtures are shown in Table 1.

Materials. Sodium caseinate was prepared from S.M.A. Co. 'Vitamin test' casein. Gelatin was Eastman Kodak purified calf-skin gelatin. Other materials used were prepared and purified by standard procedures.

L-3:5-Diiodotyrosine was provided by Prof. C. R. Harington and pteroylglutamic acid by Dr E. L. R. Stokstad, Lederle Laboratories.

RESULTS

Protein requirements of the mosquito larva

In media lacking protein or amino-acids, but complete in other respects, the mosquito larva is incapable of reaching even the second instar. Accordingly, its growth response to added protein or amino-acid mixtures is a sensitive index of the extent to which its exacting nutritional needs have been met in any particular medium. The provision of proteins in the solid state is complicated by the fact that often they cannot be maintained in finely divided form. In consequence the study of protein requirements was carried out with proteins in solution.

In order to determine the optimum concentration of protein for the mosquito larva, soluble casein was tested at various levels, with best results between 0·5 and 1%. Within this range the omission of glucose from the medium had, if anything, a beneficial effect. Lower concentrations of casein were inadequate to sustain growth (Table 2). The turbidity of soluble casein solutions after autoclaving, increasing as it did during the course of the experiment, made observation of the larvae difficult. We had recourse to sodium caseinate, which gave sparklingly clear media and at 1% concentration produced satisfactory growth and survival rates (Table 3).

Using these results as a standard of comparison, a number of other proteins and related substances were tested and it was clear that proteins known to

Table 2. *The optimum level of protein in the larval medium*

(Medium: *CM*, with changes as indicated.)

Concentration of soluble casein (%)	Concentration of glucose (%)	No. of second larval instars	No. of adult mosquitoes	Total time taken (days)	
				Mean	S.D.
0	2	0	—	—	
0·1	2	0	—	—	
0·2	0	19	0	—	
0·2	2	21	2	11·0	
0·5	0	17	11	11·5	±1·14
0·5	2	11	7	13·4	±1·21
1·0	2	33	20	11·0	±1·40
2·5	2	37	14	10·8	±1·32

Table 3. *The growth-promoting powers of various proteins*

(No. = number in stage of development indicated. The number of first-instar larvae taken was approximately 20 per experiment. Mean; S.D. = mean and standard deviation of time (in days) taken to change from previous stage of development.)

Protein tested (in solution in growth medium *CM*)	Concentration of protein (%)	Larval instars II No.	II Mean; S.D.	III No.	III Mean; S.D.	IV No.	IV Mean; S.D.	Pupae No.	Pupae Mean; S.D.	Adults Total time taken No.	Total time taken Range	Total time taken Mean; S.D.
Sodium caseinate	1·0	40	2·1±0·67	40	1·5±0·42	39	1·9±0·51	34	3·4±0·65	32	9·5–13·0	10·8±1·09
Ovalbumin	0·5	31	2·8±0·67	31	2·3±0·87	31	2·0±0·56	24	5·9±1·93	21	11·0–18·0	14·6±2·11
Gelatin	0·5	10	15·5±7·71	8	10·2±6·83	4	6·0	3	8·7	2	20·0–29·0	24·5
Gelatin	1·0	19	8·4±5·96	17	7·4±2·65	10	6·0±1·20	3	7·8	3	23·5–28·5	26·2
Edestin	1·0	20	2·6±0·69	20	2·1±0·45	20	2·4±1·09	18	5·4±1·71	17	10·5–20·5	14·3±1·93
Ovomucoid	1·0	21	4·6±1·43	20	5·3±2·52	18	6·9±3·50	10	8·8±2·19	7	18·0–33·0	22·8±4·64
Yeast nucleoprotein	1·0	14	3·3±1·48	12	1·5±0·32	12	1·9±0·30	10	4·1±0·62	10	11·0–16·5	13·1±1·72
Sericin (soluble)	0·5	0	No growth	—	—	—	—	—	—	—	—	—
Beef peptone	1–2	0	No growth	—	—	—	—	—	—	—	—	—
Tryptic digest of casein	1·0	22	4·1±1·70	22	2·8±0·78	22	2·8±0·69	20	4·1±0·77	20	13·5–18·5	15·6±1·41

be of inferior nutritive value for animals were equally unsatisfactory for the mosquito larva. Such nutritive inadequacy was revealed by decreased rates of growth and of survival (Table 3).

Casein digests and hydrolysates. With the exception of tryptic digest of casein which, as can be seen from Table 3, produced a good response, other casein digests and hydrolysates proved disappointing. Papain casein digest gave some stimulus to growth but acid hydrolysates were toxic, even after removal of chlorides and supplementation with amino-acids. In spite of all efforts to free the hydrolysates from humin and other possible toxic ingredients, they produced no growth.

Amino-acid mixtures. Preliminary studies were carried out with amino-acid mixtures used to supplement ovalbumin and gelatin. Details of the composition of these mixtures have been given in Table 1. Eleven amino-acids, in conjunction with 1% gelatin solution, produced a striking growth response (Table 4). With media containing amino-acids but no protein, one of the mixtures tested was *B*, consisting of 19 amino-acids in a concentration of 0·67%. Here growth was greatly delayed and survival poor. When the proportions were changed and the total concentration raised to 1·06% (mixture *C*) the results were more encouraging. Because of their possible toxic effects, aspartic and glutamic acids were next omitted and the amino-acid mixture *D* provided for excellent growth and survival. Mixture *I*, consisting of the same amino-acids in different proportions (more emphasis on essential amino-acids) was far less effective.

The next step was to omit amino-acids which, by analogy with requirements of higher organisms, might be expected to prove dispensable. The series of mixtures *N*, *O*, *P* and *R*, containing 14, 14, 13 and 11 amino-acids respectively in concentrations of 0·8–1·1% all gave results decidedly inferior to those obtained with mixture *D*. Mixtures *C* and *D* were also tested at one half of their previous concentrations. Although both media still contained

Table 4. *Growth of mosquito larvae on media containing mixtures of amino-acids*

Protein	Amino-acid mixture	No. of amino-acids in mixture	No. of second larval instars	No. of adult mosquitoes	Total time taken (days) Range	Total time taken (days) Mean S.D.
Ovalbumin (0·5%)	*X*	6	20	5	12·5–28	19·1
Gelatin (1·0%)	*X*	6	19	12	16·5–23	18·5±1·52
Gelatin (1·0%)	*Y*	11	21	21	10·5–15·5	12·5±1·05
Gelatin (1·0%)	*Y* without tyrosine	10	23	19	10·5–15·5	12·3±1·31
	B	19	21	10	18 –33	22·6±4·20
	C	19	20	14	10·5–16·5	13·4±1·65
	D	17	19	17	9·5–16	12·6±2·20
	I	17	22	13	16·5–26	22·7±2·83
	N	14	21	16	12 –26	15·5±3·15
	O	14	34	16	17 –29	23·3±3·67
	P	13	24	20	17 –23·5	19·3±2·56
	R	11	24	13	8·5–39·5	23·0±6·91
	C (half-strength)	19	23	13	16 –29·5	19·1±4·18
	D (half-strength)	17	18	14	14 –28	21·3±4·10

approximately 0·5% of amino-acids, growth and survival were adversely affected.

Attempts to eliminate yeast autolysate from media. In order to study the amino-acid requirements of the larva it was desirable that the basal medium should be free from such amino-acids as might be introduced by the yeast autolysate. Efforts were made to replace the autolysate by pteroylglutamic acid and the lactone of 4-pyridoxic acid which, as reported by us (Golberg & De Meillon, 1947) are capable of producing good growth in sodium caseinate media. Replacement of sodium caseinate by amino-acid mixtures such as *D* failed to produce growth, although there was no evidence of toxicity.

Attempts to concentrate the active principles from yeast autolysate by methods previously used for urine (Golberg, De Meillon & Murray, 1947) were successful only as long as caseinate was used in the larval medium, and only in the initial stages of the concentration process.

One further possibility was a reduction in the concentration of yeast autolysate, from what might appear to be the unduly high level of 2%. Since each experiment often lasted for well over a month, the deterioration of the medium in the course of prolonged incubation made it necessary to have an initially high level of the unstable growth factors.

Amino-acid requirements

With amino-acid mixture *D* as the starting point, each component amino-acid was omitted in turn. For convenience the results have been recorded separately in Tables 5 and 6, according as the amino-acids omitted proved to be 'dispensable' or 'indispensable'. The larval response was by no means as clear-cut as this division would suggest. In every case the survival rate was smaller and the rate of growth to a greater or lesser extent delayed. In some cases the tests were also carried out using as the basal medium mixture *I*, which contained much smaller proportions of 'dispensable' amino-acids. This time the omission of individual 'dispensable' amino-acids made little difference to the larval response.

In contrast to the somewhat ambiguous results with 'dispensable' amino-acids, it was quite clear that the following were quite indispensable: glycine, L-leucine, DL-isoleucine, L-histidine, L-arginine, L-

Table 5. *The effects on growth of mosquito larvae of the omission of a single amino-acid*

Amino-acid mixture	Amino-acid omitted	No. of second larval instars	No. of adult mosquitoes	Total time taken (days) Range	Mean S.D.
D	DL-Alanine	23	13	15·5–27·5	19·5±3·61
I	DL-Alanine	19	9	19·5–31·5	25·6±3·42
D	DL-Valine	21	9	13·5–25	18·7±3·0
I	DL-Valine	21	15	16 –25	19·6±2·34
D	DL-Proline	17	12	14 –21	16·5±1·89
I	DL-Proline	18	10	18 –28·5	24·5±2·07
D	L-Hydroxyproline	17	7	11 –22	13·9±3·52
I	L-Hydroxyproline	22	14	19 –30·5	25·7±3·13
D	DL-Serine	21	9	15 –18	15·7±1·25
I	DL-Serine	20	12	18·5–31	26·0±3·39
D	L-Cystine	34	15	14 –27·5	19·7±3·03
D	L-Tyrosine	20	11	16·5–24·5	20·5±2·74
D	DL-Phenylalanine	20	10	11 –19·5	13·5±2·52
N	DL-Phenylalanine	20	17	12·5–28·5	19·8±4·06

Table 6. *The effects on growth of mosquito larvae of the omission of amino-acids**

Amino-acid mixture used	Amino-acids omitted	Larval instars II No.	II Mean; S.D.	III No.	III Mean; S.D.	IV No.	IV Mean; S.D.	Pupae No.	Pupae Mean
O	Glycine	2	5·0	0	—	—	—	—	—
D	Glycine	22	7·3±1·67	22	10·5±2·12	6	7·6±1·94	0	—
O	L-Leucine	16	9·1±2·74	3	10·3	0	—	—	—
D	DL-Isoleucine	19	4·4±1·17	18	4·0±0·91	5	7·1±1·02	0	—
O	L-Histidine	0	—	—	—	—	—	—	—
O	L-Arginine	0	—	—	—	—	—	—	—
O	L-Lysine	18	9·8±3·07	12	8·7±1·89	0	—	—	—
D	L-Tryptophan	14	7·0±2·77	14	8·0±3·41	4	7·5±1·66	1	11·0
O	DL-Threonine	17	8·6±2·91	5	6·1±2·20	0	—	—	—
D	DL-Phenylalanine and L-tyrosine	0	—	—	—	—	—	—	—
D	DL-Methionine	3	11·3	0	—	—	—	—	—

* For explanation of headings see Table 3.

lysine, L-tryptophan, DL-threonine, DL-methionine. The case of DL-phenylalanine and L-tyrosine will be considered below.

The sulphur-containing amino-acids. In the experiments just described a curious phenomenon was observed in media from which cystine was omitted. Despite the presence of large amounts of DL-methionine in each medium, the great majority of the adults formed from the pupae were found dead in the medium, with only the upper half of the body projecting from the pupal case. Examination of the adults failed to reveal any obvious malformations. In addition, the growth and survival rates were reduced and could not be improved by the addition of extra amounts of methionine or DL-homocystine or glutathione. However, in the case of glutathione all the adults emerged, apparently quite normally (Table 7).

Glycine and arginine. Attempts to replace glycine by other compounds were partially successful in the case of glutathione but not when DL-serine or creatine were used. Similarly, L-arginine could be replaced to some extent by DL-citrulline, to a slight extent by L-ornithine, but not at all by creatine (Table 8).

Phenylalanine and tyrosine. In the experiments recorded in Table 5, neither L-tyrosine nor DL-phenylalanine alone could be shown to be essential, although the omission of both prevented all growth (Table 6).

Effects on cuticular pigmentation

The mosquito larva possesses chitinous plates on the head, siphon and saddle, which become more extensive as the larva approaches pupation. It is these circumscribed areas which are darkly pigmented from the second to the fourth instar, in contrast to the more diffuse pigmentation of the pupa. In most media the shade of pigmentation of the larvae varied from deep brown to jet black, but on one occasion it was noted that larvae grown on a papain liver digest medium were extraordinarily pale (Golberg, De Meillon & Lavoipierre, 1945). At the time this observation could not be repeated.

In the present series of experiments pale, unpigmented larvae were first observed in media con-

Table 7. *Effects of sulphur-containing amino-acids on the growth of mosquito larvae*

(Amino-acid mixture D used in basal medium.)

Additions to medium	No. of second larval instars	No. of adult mosquitoes	Total time taken (days) Range	Mean	S.D.
(*a*) Methionine omitted from amino-acid mixture *D*					
None	3	0	—	—	
L-Cystine (0·4 g./l.)	13	2	17–22	19·5	
L-Cystine (0·4 g./l.) + choline (0·8 g./l.)	14	1	—	19	
DL-Homocystine (0·6 g./l.) + choline (1 g./l.)	20	2	—	24	
DL-Homocystine (1·0 g./l.) + choline (1 g./l.)	8	0	—	—	
DL-Homocystine (1·0 g./l.) + choline (1 g./l.)	0	—	—	—	
(*b*) Cystine omitted from amino-acid mixture *D*					
None	34	15*	14 –27·5	19·7	±3·03
DL-Methionine (0·4 g./l.)	15	4†	17·5–24	20·0	±2·47
DL-Homocystine (0·06 g./l.)	21	12‡	15·5–33·5	21·5	±5·11
Glutathione (0·2 g./l.)	18	9	17 –25·5	21·2	±3·15

Numbers of adult mosquitoes which died while emerging: * = 11, † = 4, ‡ = 10.

Table 8. *Effect of arginine and glycine on the growth of mosquito larvae*

Compound added	No. of second larval instars	No. of adult mosquitoes	Total time taken (days) Range	Mean	S.D.
(*a*) L-Arginine omitted from amino-acid mixture *O*					
—	0	—	—	—	
Citrulline (0·6 g./l.)	21	11	16–30·5	21·4	±4·57
Ornithine (0·6 g./l.)	22	1	—	29·5	
Creatine (0·8 g./l.)	0	—	—	—	
(*b*) Glycine omitted from amino-acid mixture *O*					
—	2	0	—	—	
Serine (0·2 g./l.)	11	0	—	—	
Creatine (1·0 g./l.)	4	0	—	—	
Glutathione (1·4 g./l.)	17	5	17–26	23·7	±3·52

taining gelatin as the sole source of protein. The ghost-like appearance of the larvae was due to lack of pigment in the chitinous areas described. Supplementation with amino-acids, including phenylalanine and/or tyrosine restored the pigment. An interesting feature of these experiments was the fact that the pale larvae gave rise to pupae which were partially pigmented and, as far as could be ascertained, the adults possessed their full characteristic pigmentation. It was also noted that lack of pigmentation had little, if any, definitely adverse effect on growth or survival (Table 5).

In the case of amino-acid mixture *D* the omission of tyrosine produced pale larvae and almost completely unpigmented pupae, but the omission of phenylalanine appeared not to affect pigmentation. This apparent paradox was resolved by increasing the level of phenylalanine in the tyrosine-free medium. At a level of 1·0 g. DL-phenylalanine/l. of medium the larvae were pale up to the third instar, but the fourth-instar larvae were dark and the pupae appeared normal. At a level of 1·4 g. of DL-phenylalanine pigmentation was normal throughout.

Intermediate degrees of pigmentation. Observation of fourth-stage larval pelts or, where these were not available, of early instar larvae, indicated the existence of the following intermediate degrees of pigmentation between the colourless larva and the dark normal shade:

0 White, with no pigment visible.
1 Siphon and saddle clear yellow; no darkening.
2 Siphon and saddle bright yellow; no darkening.
3 Slight darkening of siphon and saddle.
4 Siphon definitely darkened; saddle with a large, clearly marked area dorsally.

It was thus a matter of interest to us to assess the shades of pigmentation resulting from the use of various possible precursors of melanin in a basal medium capable of producing colourless larvae but fairly good growth and survival. Some of the compounds tested were too toxic to permit growth to the fourth instar, but the remainder gave rise to varying degrees of pigmentation (Table 9).

DISCUSSION

Insects offer a striking contrast to higher animals in that many can dispense with dietary nitrogen during adult life (Uvarov, 1928). The proteins utilized during this time must be drawn from reserves accumulated during larval life. An additional factor to be taken into consideration in many instances is the rapid rate of growth. These factors operating together ensure that the mosquito larva is highly exacting with regard to its protein requirements. Failure to obtain protein prevents growth even to the second instar. Failure to obtain protein of adequate quality may have the same effect or may prolong the time of development far beyond its normal span.

For optimum growth in our media the mosquito larva requires a level of protein as high as 1 % and, apparently, equally high concentrations of total amino-acids. In earlier experiments (Golberg *et al.* 1945) we had found that a very light autoclaved suspension of micro-organisms sufficed for excellent growth of the larvae. In fact, it was frequently observed in contaminated media that before the presence of the contaminants was visible to the naked eye it was betrayed by a distinct acceleration of larval growth. The high level of protein and of amino-acids found necessary in our experiments may be a consequence of the nature of the media employed or of the fact that the mosquito larva is equipped physiologically to deal with discrete particles rather than solutions.

In this connexion attention should be drawn to the remarkably high levels of amino-acid nitrogen observed in the blood of insect larvae and chrysalids (Bishop, Briggs & Ronzoni, 1925; Courtois, 1928; Duval, Portier & Courtois, 1928). Values as high as 4 g. amino-nitrogen/l. have been reported, corresponding to well over 20 g. of amino-acids/l. of haemolymph. Such a consequence of histolytic changes probably obtains in the mosquito larva and would explain the necessity for the high level of dissolved protein or amino-acids.

Table 9. *Pigmentation induced in mosquito larvae by various compounds*

(Medium contained 0·5 % gelatin and amino-acid mixture *Y* with tyrosine and phenylalanine omitted. All compounds tested at a concentration of 0·4 g./l.)

Compound tested	Specimens examined	Degree of pigmentation*
DL-Phenylalanine	Fourth-stage pelts	1
L-Tyrosine	Fourth-stage pelts	4
Tyramine hydrochloride	Fourth-stage pelts	1
L-3-Aminotyrosine	Fourth-stage pelts	2
L-3:4-Dihydroxyphenylalanine	Third-stage larvae	0
DL-α-Aminophenylacetic acid	Fourth-stage pelts	1
p-Aminophenylacetic acid	Fourth-stage pelts	2–3
L-3:5-Diiodotyrosine	Third-stage larvae	0
Adrenaline	Second-stage larvae	0

* The significance of the numbers used is explained in the text, p. 384.

The mosquito larva is exacting in its requirement for 'complete' proteins. In this respect it resembles *Drosophila* (Lafon, 1938), *Blatella* (McCay, 1933) and *Pyrausta* (Bottger, 1942). Lafon & Teissier (1939) have drawn attention to the suitability of yeast proteins for the nutrition of *Tenebrio*, and their remarks apply to many other insects, including the mosquito larva.

Our inability to use casein hydrolysates for the mosquito larva recalls a similar experience reported by van't Hoog (1935) with *Drosophila*. Lafon (1938) and Tatum (1941) met the protein needs of *Drosophila* by means of casein hydrolysates supplemented by cystine and/or tryptophan. On the other hand, Kozhanchikov (1944), Lafon (1938) and Buddington (1941) met with little or no success in their efforts to replace proteins by amino-acids in insect diets. Our results with mixtures of amino-acids show clearly that it is not only the qualitative but also the quantitative composition of an amino-acid mixture which determines its growth-stimulating ability. By demonstrating the improvement in the rates of growth and survival which is brought about by the presence of non-essential amino-acids in adequate proportions these experiments lay emphasis on the antagonistic and toxic effects of amino-acids (Gladstone, 1939; Hutchings & Peterson, 1943; Pelczar & Porter, 1943; Porter & Meyers, 1945).

The suggestion that strepogenin (Woolley, 1946 and earlier references) is required by the mosquito larva would serve to explain the fact that growth on amino-acid mixtures is in general inferior to that which results from the use of casein; and that pteroylglutamic acid and the lactone of 4-pyridoxic acid can produce growth with sodium caseinate but not with amino-acids. However, proteins such as egg albumin and gelatin, known to contain little strepogenin (Sprince & Woolley, 1945), produced slow growth; and when supplemented with amino-acid mixture *Y*, gelatin led to growth not greatly inferior to that obtained with casein.

Essential amino-acids. In connexion with larval requirements of amino-acids the concept of 'essentiality' has a special significance, since provision must be made not only for growth but at the same time for maintenance of the adult. It is interesting to observe, however, how closely the requirements of the larva resemble those of higher animals, in particular those of the chick. Thus glycine and arginine are indispensable amino-acids for the chick (Klose, Stokstad & Almquist, 1938; Almquist & Mecchi, 1940; Hegsted, Hier, Elvehjem & Hart, 1941); they are also indispensable for the mosquito larva. The chick can utilize citrulline but not ornithine in place of arginine (Klose & Almquist, 1940; Klose *et al.* 1938). The mosquito larva is able to grow and metamorphose when given citrulline; it does utilize ornithine to a small extent, since there was delayed growth to the fourth instar. (The maximum times recorded were: to the second stage, 18·5 days; to the third, 20 days; to the fourth, 15 days; to the pupa, 12 days. Many larvae were still alive when the experiment was discontinued.) There is a striking similarity between these observations and the growth of *Streptococcus haemolyticus* on a medium in which arginine was replaced by ornithine: Gale (1945) reported that with ornithine growth was only one quarter of that with arginine and was also irregular. Although isotope experiments have demonstrated the conversion of L-serine into glycine in the rat and guinea pig (Shemin, 1946), Almquist & Grau (1944) found that the omission or inclusion of serine in chick diets had no effect on their need for glycine. Similarly, the mosquito larva was unable to utilize serine in place of glycine. With glutathione its response was definite but delayed and incomplete. In contrast to its effectiveness in the chick (Almquist, Mecchi & Kratzer, 1941; Hegsted *et al.* 1941) creatine proved incapable of replacing glycine in the larval diet.

Little is known concerning the role of glycine in invertebrates. Kutscher & Ackermann (1933) noted the surprising fact that glycine betaine has not been found in insects, although it definitely occurs in Crustacea. They attributed this observation to the rapidity of insect metabolism, which prevents the accumulation of glycine in appreciable quantity and hence the formation of betaine.

Valine. Microbiological assay of valine using *Lactobacillus arabinosus* revealed a concentration of 29 mg. valine per g. of yeast autolysate. It is doubtful whether in such a case the entire response of the test organism was due to valine, but the result accounts for the finding that growth of the mosquito larva is not seriously affected by lack of added valine in the medium. A final decision on the essentiality of valine for the mosquito larva will have to be deferred until the existing difficulties in devising a suitable test medium have been overcome.

Sulphur-containing amino-acids. That cystine is a dispensable amino-acid is generally accepted. So much so, that most investigators of recent years have made no effort to ascertain whether in fact cystine is replaceable in all its functions by methionine. Hegsted (1944) acknowledged this omission in studies on the amino-acid requirements of chicks. There exists considerable evidence, especially among micro-organisms, that cystine plays an indispensable part in nutrition in certain circumstances. Such observations give added point to the effect of cystine lack on the mosquito larva. The failure of a large proportion of adults to emerge successfully suggests that a deficiency of cystine is possible, despite the high level of methionine in the medium. Moreover, while cystine and homocystine are both capable of replacing methionine to some extent, only gluta-

thione could replace cystine in its ability to promote normal emergence of adults.

Phenylalanine and tyrosine. Mosquito larvae seem equally capable of utilizing either phenylalanine or tyrosine, a fact which might be explained by the presence of a small quantity of phenylalanine in the yeast autolysate used. This suggestion fails to account for the fact that growth is delayed in the absence of tyrosine but remains unaffected when phenylalanine is omitted from the medium.

The production of unpigmented larvae on media lacking tyrosine or phenylalanine is, to our knowledge, the first demonstration that the absence of these amino-acids from the diet can affect pigmentation. It is instructive to consider the quantitative aspect of these experiments. Since all media contained equal amounts of yeast autolysate, this factor remained constant. From the values given by Hodson & Krueger (1946), a 1 % solution of sodium caseinate would contain 0·48 g. L-phenylalanine and 0·53 g. L-tyrosine/l. This medium produces full pigmentation. In an amino-acid medium, DL-phenylalanine, in a concentration of 1·4 g./l., also produces darkly pigmented larvae; but a level of 1·0 g./l. allows pigmentation of the fourth-stage larva but not of the earlier instars; 0·6 g./l. produced no visible pigmentation. In 0·5 % gelatin the amount of phenylalanine present would be 0·13 g./l. (calculated from the analysis of Block, Jervis, Bolling & Webb, 1940). An additional 0·4 g./l. of L-tyrosine is sufficient for the dark pigmentation of fourth instar larvae; but the same amount of DL-phenylalanine produces pale yellow pigmentation. Thus both with amino-acid mixture *D* and with gelatin it is clear that L-tyrosine is greatly superior to DL-phenylalanine in its ability to promote the formation of melanin. It may be that D-phenylalanine is not utilizable for melanin formation—*in vivo* only L-3:4-dihydroxyphenylalanine is acted upon by dopa oxidase (Sumner & Somers, 1943). Taking into account the strong melanin-forming powers of *p*-aminophenylacetic acid (Table 9) it seems more probable that DL-phenylalanine is utilized for the most part for other, more essential, purposes.

The activity of *p*-aminophenylacetic acid, and even to a small extent of α-aminophenylacetic acid, indicates that compounds of various types must be taken into consideration as precursors of melanin *in vivo*. The findings of Pryor, Russell & Todd (1946) that the phenolic substance responsible for the hardening of the cockroach ootheca is protocatechuic acid, in addition to the results of earlier workers, suggests that a wide range of compounds may possibly play a part in the darkening of the insect cuticle.

Serra (1946) has shown that melanins extracted from hairs of different hues contain varying proportions of melanoid pigment and protein. According to his analyses, a combination of 60 % melanoid pigment and 40 % protein leads to a black melanoprotein, but 30 % pigment and 70 % protein constitutes a yellow melanoprotein. The colours observed in the head, siphon and saddle of the mosquito larva are therefore indicative of the relative proportions of melanoid pigment formed under the experimental conditions employed. From our results it becomes clear that the production of melanin is a process depending directly on the level of dietary phenylalanine and tyrosine—or, more correctly, on the excess over the quantities required for growth and protein synthesis. In consequence of this fact, varying degrees of melanic pigmentation can be produced at will and absence of pigmentation during the early instars, when all available phenylalanine is presumably required for protein synthesis, can be followed by the production of some melanin when the larva is full-grown.

Our observation that in the mosquito larva pigmentation has little connexion with growth underlines the statement made by Wigglesworth (1942) that 'the pigmented constituents of insects, in some cases...are, perhaps, substances of physiological importance; but the majority seem to be merely by-products of metabolism'. Since tanning by means of a melanin precursor plays an essential part in the hardening of the insect cuticle (Pryor, 1940; Trim, 1941; Hurst, 1940, 1945) it is possible that in unpigmented larvae the cuticle remains soft. The physiological implications cannot be discussed here. There is obviously a wide scope for the use of the nutritional approach to the study of melanin formation *in vivo* for the elucidation of the many problems as yet unsolved in this field.

SUMMARY

1. In a medium complete in other respects, but lacking protein the mosquito larva does not grow to the second instar.

2. After yeast, casein is the most suitable protein, but incomplete proteins, on supplementation with amino-acids, prove adequate for growth and survival to the adult stage.

3. Good rates of growth and survival could be produced by using mixtures of amino-acids, but variation in their relative proportions or reduction in the number of amino-acids had adverse effects.

4. By omitting single amino-acids from a mixture, it was possible to establish that the following are essential for the mosquito larva: glycine, L-leucine, DL-isoleucine, L-histidine, L-arginine, L-lysine, L-tryptophan, DL-threonine, DL-phenylalanine and DL-methionine. The status of DL-valine could not be determined while using yeast autolysate in the medium.

5. There was evidence that the omission of L-cystine resulted in a high proportion of adult mosquitoes failing to emerge.

6. According to the level of phenylalanine or tyrosine in the medium various shades of pigmentation could be produced in the mosquito larvae. The adults emerging even from wholly unpigmented larvae were normally pigmented and there appeared to be little relation between pigmentation and growth or survival. A number of compounds were found capable of producing intermediate degrees of pigmentation in the mosquito larva.

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The Chemistry of Connective Tissues

1. THE STATE OF COMBINATION OF CHONDROITIN SULPHATE IN CARTILAGE

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In recent years evidence has been presented by several groups of workers that chondroitin sulphate exists in tissues such as cartilage in a very highly polymerized condition. The early workers in the field regarded chondroitin sulphate as an oligosaccharide of quite low molecular weight, and Levene (1925) formulated the substance as a non-reducing tetrasaccharide composed of two residues of glucuronic acid and two residues of a sulphate of *N*-acetylglucosamine. Perhaps the main reason for the failure of the early workers to recognize the highly polymerized character of the polysaccharide lay in the fact that strongly alkaline reagents were invariably used to effect the initial extraction from the tissue, and it has since been shown that the polysaccharide is rapidly degraded in the presence of strong alkali, particularly at temperatures above 0°.

The quantitative extraction of chondroitin sulphate from cartilage remains a matter of considerable difficulty, and dilute NaOH is still frequently employed. This method was investigated by Jorpes (1929), who showed that a concentration of NaOH as high as 0·5 N was necessary to extract the acid polysaccharide from cartilage at 0°. Meyer & Smyth (1937) suggested the use of strong solutions of calcium, strontium or barium chlorides, and obtained good yields of chondroitin sulphate by the extraction of dried cartilage powder with 10% (w/v) $CaCl_2$ solution at neutral reaction.

Blix & Snellman (1945) used $CaCl_2$ extraction and were careful to avoid acids or alkalis during the subsequent separation of the polysaccharide from the protein moiety of the extracted mucoid. Prepared under careful conditions, the chondroitin sulphate was shown to have a molecular weight of the order of 260,000 and a long-chain structure was suggested. In dilute alkali, the molecule was depolymerized at room temperature to a rather definite point, at which the products formed were still of colloidal size, although giving little double refraction of flow. The molecular weight of chondroitin sulphate extracted from cartilage by dilute alkali was estimated to be of the order of 40,000–50,000.

Meyer & Odier (1946) prepared chondroitin sulphate by extraction of hog nasal cartilage in 0·5 N-NaOH at 5°. Their preparation was probably similar to the alkali-degraded polysaccharide of Blix & Snellman (1945), and from viscosity measurements calculated on the basis of an unbranched chain its molecular weight was estimated at 10,000–15,000. The reducing power of the polysaccharide towards NaIO at pH 10·5 and towards HIO_4 at pH 4·2–4·4 was investigated, and from the results obtained it was concluded that the residues of glucuronic acid and *N*-acetylglucosamine-6-sulphate are arranged to form an unbranched chain molecule. This conclusion is in opposition to the earlier view of Bray, Gregory & Stacey (1944) who methylated a degraded chondroitin that had been obtained by acid hydrolysis of a chondroitin sulphate prepared with alkali. From isolation of the products of hydrolysis of the methylated oligosaccharide it was concluded that chondroitin sulphate possesses a structure of the branched-chain type.

The chondroitin sulphate of hyaline cartilage is associated with the semi-transparent, apparently structureless, ground substance that constitutes the bulk of the tissue and gives the cartilage its characteristic physical properties. The cartilage cells occupy cavities in this ground substance and appear to be isolated from one another. In spite of its apparent homogeneity, histological studies show that the intercellular ground substance is not amorphous since after digestion with trypsin or by use of the various silver impregnation methods, collagenous fibrils are discernible which form a dense felt-work running in all directions or gathered together in oriented bundles. From its histochemical reactions the collagen of the ground substance of cartilage is considered to be similar to that occurring in loose connective tissue. When cartilage is extracted with alkalis or with solutions of inorganic salts, part of the material passes into solution and constitutes the so-called 'chondromucoid'; the residue is mainly collagen but retains more or less mucoid according to the efficiency of the agent used for extraction. The chondromucoid contains protein and gives precipitates on addition of acetic acid which usually take the form of clear firm gels.

The nature of the protein moiety of chondromucoid and its relation to the chondroitin sulphate and the collagen structures of the tissue itself have received relatively little attention. Bungenberg de Jong & Dekker (1935) studied the interaction of chondroitin sulphate with proteins and showed the presence of complexes which were regarded as 'coacervates'. Meyer, Palmer & Smyth (1937), on the other hand, looked upon the interaction as a salt formation and showed that in the presence of acetic acid true salts are formed in stoicheiometric proportions by the union of the basic groups of the protein with the acid groups of the polysaccharide. It was shown that in the presence of 2% (w/v) acetic acid, the amount of chondroitin sulphate combined by a protein corresponded to the amount of basic amino-acids contained in the protein, and further, the chondroitin sulphate-binding power of the protein was in agreement with the acid-binding power found by studies of dye-protein complexes. In the salts studied the chondroitin sulphate was found to react with the protein as a dibasic acid; both the sulphuric acid group and the carboxyl group of the uronic acid residue take part in salt formation. Meyer *et al.* (1937) were of the opinion that chondroitin sulphate occurs in nature in salt-like complexes which are present as structurally organized elements of sheet or fibre form, and pointed out that analysis of ground cartilage showed the ratio of hexosamine N to total N to be 0·046, a figure that compares well with the corresponding ratio 0·056 for the gelatin salt of chondroitin sulphate. This close correlation led to the view that the major portion of the cartilage is a protein salt of chondroitin sulphate.

This suggestion is not borne out by the experiments of Blix (1940), who submitted an aqueous extract of cartilage (nasal septum of ox) to electrophoresis in the apparatus of Tiselius (1937) at hydrogen-ion concentrations within the physiological range. Two components only appeared, the faster being almost pure chondroitin sulphate while the slower was a protein containing only 0·02% hexosamine. No evidence of combination at a higher pH than the isoelectric point of the protein was reported, and the result appears to support the earlier view of Mörner (1889) that in cartilage the acid polysaccharide is at least partly present as alkali salt and is, therefore, not fully combined with protein. It should be pointed out, however, that only a very small proportion of the chondroitin sulphate is extractable from fresh cartilage by water under the conditions used by Blix, and the extracted mucoid may not be in the same form as the material remaining in the tissue.

The intention of the work described here was to make a preliminary study of the chemical structure of the intact tissue, and in embarking on this problem it was considered that a possible line of approach would lie in the application of a process of successive mild degradation of one or other of the components of the tissue, followed by an examination of the breakdown products that resulted. The process of degradation of collagen fibres as they occur in tendon or hide has already received much attention, particularly by workers interested in the mechanical properties of leather, and for this reason the first approach has been a study of the liberation of chondromucoid under conditions that would be expected to give rise to thermal degradation of the collagen structures present in the tissue.

The work of Lloyd & Garrod (1946) has shown that collagen fibres of tendons (from the tails of rats) rapidly contract to about one third of their original length when heated in water to 60–70°, and that in the contracted state the fibres show a new property of rubber-like elastic extension. Lloyd and her colleagues found that formamide, strong solutions of the phenols and anhydrous formic acid are very effective in reducing the temperature at which thermal contraction takes place. Strongly acid (pH 2) or alkaline (pH 12) solutions and concentrated solutions of salts are also effective. Among the salts the effect of the lyotropic series is evident and, in addition, Ca^{++} is stated to be particularly effective in causing shortening in length at low temperatures.

Since published data on the thermal contraction of collagen fibres were insufficient to afford a direct comparison with the methods devised for the extraction of cartilage, a number of experiments were carried out during the course of this work to determine the effect on rat-tail tendon of the temperature and concentration conditions used in the extraction experiments, and these are reported in the experimental section.

It is probable that the chondroitin sulphate liberated under the rather mild conditions that give rise to thermal shrinking of collagen fibres remains in a condition closely approaching its state in the tissue itself and, with this in mind, it was considered that a study of the physical and chemical properties of the extracted mucoid would be of value. Evidence of complex formation between the undegraded polysaccharide and the protein of the mucoid under conditions of pH and salt concentration in the physiological range is of particular interest, and for this reason a preliminary electrophoretic study of the material extracted by the new method has been undertaken.

EXPERIMENTAL

Analytical methods

A modification (Lugg, 1938) of the peroxide fusion method was used for the estimation of total S. Ester-bound S was determined as SO_4-S after hydrolysis with 5 N-HCl for 20 hr. at 100°, using the gravimetric procedure described by Lugg (1938). The micro-Kjeldahl method was used for the determination of N. Moisture was determined by drying to constant weight over P_2O_5 at 80° under reduced pressure, and in all cases ash was weighed as sulphate.

All analyses quoted in the text are corrected for ash and water unless it is otherwise stated.

Preparation of the cartilage powder

Fresh cartilage from septum nasi of cattle was freed from adhering connective tissue and finely chopped with a scalpel. The pieces were introduced at once into acetone, and after standing for some time in two or three changes of solvent they were dried in air at 70° and ground in a hammer mill through a sieve with circular apertures 0·56 mm. in diameter.

Extraction of cartilage powder with water at room temperature

Although little mucoid could be extracted from fresh cartilage by water at room temperature, even after the tissue had been finely minced, a considerable proportion of the mucoid became extractable after the tissue had been dehydrated in acetone and ground to a powder. Portions of the powdered cartilage (N, 12·4; ester S, 1·2; P, 0·06; ash, 5·6; moisture, 5·6 %) were extracted with distilled water by shaking gently for 2–3 hr. at room temperature. One extraction was sufficient to remove the bulk of the extractable material, but in order to exhaust the residue, two further similar treatments were given. The chondromucoid was precipitated from the aqueous solution by addition of ethanol (3 vol.) after adding a few ml. of saturated sodium acetate solution to bring the concentration of the salt to about 0·5 % (w/v). The precipitate was washed first with 80 % (v/v) ethanol and then thoroughly dehydrated with anhydrous ethanol before drying. Prepared in this way the mucoid was a fine white powder having N, 9·2–11·5; ester S, 3·2–3·3; P, 0·04 %; and dissolved in water to give a clear viscous solution. Results obtained from a number of experiments showed that 30–35 % of the acid-hydrolyzable S present in the original tissue was extractable by distilled water at 18–23°.

Effect of a short heat treatment on the extraction of cartilage powder

Cartilage powder that had been thoroughly extracted with water at room temperature yielded a further amount of chondromucoid after a short heat treatment in water at 60–80°. Portions of the water-extracted powder (1·55 g.) yielded 0·273 g. of chondromucoid after heating with distilled water at 80° for 10 min., followed by shaking gently for 2 hr. at room temperature. In a subsequent series of experiments it was found that a preparation of cartilage powder that had already yielded 35 % of its content of ester S by extraction with cold water yielded a further 30–35 % of the ester S originally present in the tissue, on treatment with distilled water at 60° for 30 min. The chondromucoid extracted after heat treatment was similar in composition to that extracted by cold water having N, 10·7–12·4; ester S, 3·1–3·7 %. The material remaining insoluble still contained 33–36 % of its original content of ester S, having N, 14·7–15·0; ester S, 0·6–0·63 %.

Extraction with calcium chloride solution

Extraction with a 10 % (w/v) aqueous solution of $CaCl_2$ was found to be more effective after an initial short period of gentle heating. A sample of cartilage

powder that had already been exhaustively extracted with cold water was warmed to 45° for 30 min. with 10 % (w/v) $CaCl_2$ solution (pH 7·49), followed by cooling to room temperature and shaking for 3 hr. The extracted chondromucoid had N, 8·2; ester S, 3·8 % and represented 28 % of the ester S originally present in the fresh cartilage. The substance after recovery from the $CaCl_2$ solution was not entirely soluble in distilled water or in dilute salt solutions, and gave a small amount of gelatinous residue. After removal of insoluble material by centrifugation, the aqueous solution was clear and viscous. Since extraction with neutral $CaCl_2$ solution under these conditions was incomplete, the extraction was repeated with a solution of $CaCl_2$ (10 % w/v) that had been adjusted to pH 11 by addition of $Ca(OH)_2$ followed by filtration. The mixture was heated to 37° for 30 min., and in this case a total extraction of 90 % of the ester S was obtained, but the product (N, 11·7; ester S, 3·6 %) did not give highly viscous aqueous solutions and appeared to be more readily soluble in water than the mucoid extracted at neutrality. The residue from the cartilage after a further extraction with the alkaline $CaCl_2$ reagent had N, 16·5; ester S, 0·15; total S, 0·56 %, and since all but a small residue (6·3 %) was converted to gelatin on heating with water at 120°, the substance was considered to be almost wholly collagen.

Extraction with aqueous solutions of formamide

Freshly prepared solutions of anhydrous formamide (m.p. −2°) were used for these experiments. Neither 10 % (v/v) nor 30 % (v/v) solutions were effective in extracting further mucoid from water-extracted cartilage powder at temperatures up to 37°. However, on warming to 45° for 30 min. a further 30 % of the ester S originally present in the tissue was extracted by 30 % (v/v) formamide solution, and the product after recovery by precipitation with ethanol (N, 10·0; ester S, 3·3 %) was similar in analytical composition to the product of extraction with hot water or calcium chloride solutions. The mucoid extracted with 30 % (v/v) formamide, redissolved in water to give clear viscous solutions.

Thermal contraction of tendons from the tail of the rat

The procedure adopted was a modification of that of Lloyd & Garrod (1946). The tendons were carefully extracted from rat tails that had been stored for 2 weeks at 0°, and any tendon that had been stretched or damaged in the process of removing it from the tail was rejected. To both ends of each tendon a small lead shot was attached by inserting the end of the tendon into a deep cut in the shot, made by a razor blade, and then gently closing the cleft by means of a pair of forceps.

Table 1. *Thermal contraction of rat-tail tendon*

(The length of the tendon at the time indicated is expressed as percentage of the initial length.)

Temp.	2 min.	5 min.	10 min.	20 min.	60 min.
		Solvent: distilled water			
55°	100	100	100	100	100
60	49	64	95	97	100
65	47	93	100	103	110
70	33	104	108	112	116
		Solvent: 10 % (w/v) $CaCl_2$ (pH 6·05)			
37°	100	100	100	98	92
45	—	60	53	90	120
50	43	68	88	100	106
		Solvent: 10 % (w/v) $CaCl_2$ (pH 11·2)			
40°	—	86	27	30	116
45	—	27	27	44	111
50	23	23	36	84	118
		Solvent: anhydrous formamide			
35°	—	95	67	67	95
40	—	67	69	100	102
45	—	61	100	110	112
		Solvent: 30 % (v/v) aqueous formamide			
30°	100	100	100	97	35
40	67	76	95	102	106
45	57	57	66	74	77
		Solvent: 10 % (v/v) aqueous formamide			
40°	—	87	27	30	110
45	—	27	27	44	87
50	23	23	36	84	118

The tendon was suspended in a narrow graduated cylinder which contained the solvent, the upper lead shot being gripped in a clip while the lower one hung free. The weight of the lower shot was chosen to be just sufficient to sink the tendon and hold it at full length. The graduations on the cylinder were used as an arbitrary unit for measuring the changes in length of the tendon, and for temperature control the cylinder was placed in a water bath fitted with a stirrer.

The results of a number of experiments are given in Table 1, which shows the changes in length with time at various temperatures and in various solvents. In all experiments, little change in length occurred below a certain limiting temperature; above this temperature, however, rapid contraction took place and this was followed by a slow increase in length so that the final length of the tendon was often greater than the initial value. At this stage the tendon had lost much of its tensile strength and had become partially gelatinous. In the table, only those experiments carried out near the limiting temperature are reported. It should be noted that there was some variation in the behaviour of individual tendons, even among those extracted from the same rat's tail, but the values recorded are typical of the results obtained from many experiments.

Correlation of the conditions resulting in extraction of chondromucoid with those giving rise to thermal contraction in collagen fibres

In Table 2 the results of a number of extraction experiments are given. The sample of cartilage powder used had not been previously extracted with water at room temperature so that the yields given (expressed as percentage ester S extracted from the original tissue) are total yields. It seems clear that a short period of heating materially assists the release of chondromucoid from the remaining constituents of the tissue, either in aqueous solution or in solutions of formamide or salts. Further, the action of $CaCl_2$ or formamide solutions is not due entirely to an enhanced solubility of the chondromucoid in these reagents, since the substance is readily soluble in cold water, and can in fact be extracted in good yield by distilled water alone after a short heat treatment. Solutions of the active reagents have, however, a marked effect in reducing the temperature of the heat treatment necessary to effect extraction. Comparison of Table 2 with Table 1 shows the marked correlation between the reaction conditions necessary to extract chondromucoid in good yield with those giving rise to thermal contraction of free collagen fibres. It should be pointed out, however, that, except in the experiments with $CaCl_2$ solution at pH 11, complete extraction was not obtained, and in this one case there is reason to suppose that the mucoid itself suffered degradation since the product gave solutions that were considerably less viscous than those obtained from chondromucoid extracted with less alkaline media. Although there were considerable variations in the N and S contents of the extracted mucoids in individual experiments, the figures given in Table 2, which show the range obtained over a number of duplicate experiments, indicate little systematic variation and show that the product extracted under the varying conditions had substantially the same analytical composition.

Isolation of the protein component

Investigations on mucopolysaccharides of bacterial origin have shown that the solubility of the polysaccharide moiety of the complexes in strong solutions of phenol is in some cases less than that of the protein (cf. Morgan & Partridge, 1941). In the case of chondromucoid it was found that at 37° considerable amounts of protein may be extracted from the

Table 2. *Effect of heat treatment on the extraction of cartilage powder in various solvents*

Treatment	Ester S extracted (%)	Chondromucoid	
		Ester S (%)	N (%)
Water at 20°	30–35	3·15–3·3	9·2–11·5
Water at 60°, 30 min.	58	3·1	12·4
Water at 80°, 10 min.	65–70	3·5–3·7	10·7–11·0
$CaCl_2$ (pH 7·5) 45°, 30 min.	60	3·5–3·8	8·2–9·7
$CaCl_2$ (pH 7·5) 55°, 30 min.	65	3·5–3·6	9·3
$CaCl_2$ (pH 11) 37°, 30 min.	95–98	3·6–3·8	11·7
Formamide 30% (v/v) 37°, 30 min.	44	—	—
Formamide 30% (v/v) 45°, 30 min.	63	3·3	10·0

dry powder by 90% (w/w) aqueous phenol. The mucoid (2 g.; N, 9·41; ester S, 2·33; ash, 9·3; moisture, 6·5%) was allowed to stand at 37° with 90% (w/w) phenol solution (150 ml.) for 24 hr., the mixture being occasionally stirred. The residue was removed by centrifugation and the clear solution treated with ethanol (3 vol.). The heavy precipitate, after collecting and washing with ethanol in the centrifuge, was dissolved in water at 60° and again precipitated with ethanol. After washing with ethanol and ether the substance was dried at room temperature over P_2O_5.

Prepared in this way, the substance had N, 17·53; ester S, 0·13; total S, 0·44% and was thus essentially protein in character. The residue from phenol extraction had N, 7·96% (uncorrected for ash and water) and was extracted with further quantities of 90% phenol solution. The yield of protein from the first extract was 0·2 g., but from successive extracts the amount of protein yielded diminished rapidly and after four extractions the N content of the residue remained constant at 7·26% (uncorrected). It was clear, therefore, that although part of the protein contained in the 'undegraded' mucoid was freely extracted by phenol solution, a further part of it was not available for extraction, and in subsequent experiments it was not found possible to reduce the N content of the mucoid below about 7% by repeated extraction with phenol. The protein was not soluble in cold water or cold dilute alkalis or acids, but dissolved readily in water at 50–60° giving clear viscous solutions. On cooling, the solutions set to a firm gel.

Viscosity of the extracts

The dry preparations of chondromucoid were usually not completely soluble in water or buffer solutions, and solubility slowly decreased on storage in the dry state. Solutions of the chondromucoid, on the other hand, were fairly stable, but it was observed from time to time that the viscous solutions slowly became more opalescent on standing at 0° and sometimes gave rise to a fine precipitate.

These effects caused difficulty in making up solutions of the mucoid for viscosity determinations or for electrophoresis experiments and for this reason fractionation with ethanol was eliminated and the extracts obtained in solutions of electrolytes were first dialyzed against distilled water for 48 hr. followed by dialysis against the appropriate buffer solution. Concentration was then measured refractometrically by making use of a value for the refractive increment of the mucoid in the buffer solution. Since the composition of the mucoid was rather variable, particularly as regards ash and N content, there was some uncertainty about the value of the refractive increment to be adopted, but from a number of experimental determinations on mucoid extracted from the cartilage powder in phosphate-NaCl buffer of pH 6·90 at 60° an average value of $1·60 \times 10^{-3}$ (1% (w/v) at 25·2°) was adopted for all the refractometric estimations.

Viscosity determinations were carried out at 25·4° in an Ostwald viscosimeter having a capillary length of about 9 cm. and a flow-time for water of 31·2 sec. As would be expected the relative viscosity (η_r) of the mucoid in salt solution was much lower than that given by the same preparation in distilled water; for instance, mucoid that had been extracted by distilled water at 60° gave (after dialysis against changes of distilled water) a value for η_r in water of 3·16 for a 0·25% solution, while after dialysis against phosphate-NaCl buffer of pH 6·97 (μ, phosphate, 0·02; μ, NaCl, 0·18) the solution had η_r 1·53 at the same concentration. The viscosity of the mucoid, both in water and salt solutions, showed variations from preparation to preparation even under carefully controlled conditions of extraction, but the values for the viscosity of individual preparations at different concentrations lay on smooth curves. The values for log η_r for different dilutions of most of the preparations could be fitted by straight lines, and Fig. 1 shows log η_r plotted against concentration for a number of the preparations.

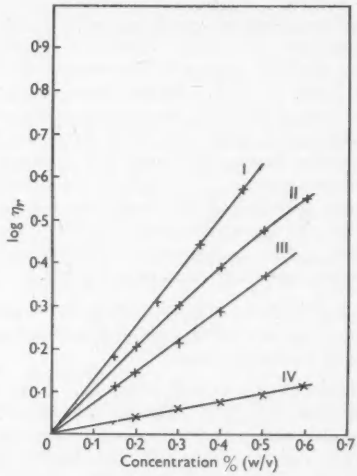

Fig. 1. Log η_r for extracts of cartilage, viscosity determinations carried out in phosphate-NaCl buffer of pH 6·90; μ, phosphate, 0·02; μ, NaCl, 0·20. I, H_2O at 70°; II, $CaCl_2$ (pH 7·4) at 55°; III, Phosphate-NaCl at 60°; IV, $CaCl_2$ (pH 11·0) at 37°.

If the equation of the log η_r-concentration curve is taken as

$$\log \eta_r = Kc,$$

where K is a constant and c is the concentration of the solute, then, at infinite dilution (provided natural logs are used), k = the 'intrinsic viscosity' $[\eta]$, where

$$[\eta] = \lim_{c \to 0} \frac{(\eta_r - 1)}{(c)} \text{ (Kraemer \& Lansing, 1935).}$$

According to Staudinger (1940) the value of intrinsic viscosity is proportional to particle weight even in the case of heteropolar substances, provided that the particles are linear macromolecules and charge effects are suppressed by carrying out the viscosity determinations in solutions containing a sufficient concentration of electrolyte. However, the solutions of mucoid contain more than one molecular species and, as will appear later, in dilute solutions containing electrolytes chondromucoid may be looked upon as a complex in equilibrium with its dissociation products. Under these conditions it is probable that the significance of $[\eta]$ as a measure of particle length is qualitative only, but since $[\eta]$ for the extracts made under alkaline conditions is of a lower order compared with the values given by the mucoid extracted at neutral reaction, it is clear that in the former case considerable degradation has occurred.

It is implicit in the theory of Staudinger that the effect of molecular association on viscosity depends upon the relative position of the molecules associating together. If compact bundles are formed, the viscosity is supposed not to be affected, while, if two similar molecules associate end to end, the value for $[\eta]$ is twice as great as in the absence of association. The linearity of the log η_r curves is therefore of especial interest since it indicates that, in the presence of electrolytes, the degree of end to end association is not significantly increased with increasing concentration.

Effect of heat treatment on the viscosity of chondromucoid

An extract of chondromucoid was prepared in phosphate-NaCl buffer of pH 6·9 (μ, phosphate, 0·02; μ, NaCl, 0·18) and was dialyzed against several changes of the same buffer. Samples of the solution (10 ml.; *c.* 0·481 % (w/v)) were sealed in glass tubes and heated for varying periods of time, one series being heated at 100° and another at 80°. At both temperatures the viscosity of the solutions fell very rapidly in the first 5 min. of heating, the initial rapid fall being followed by a further slow reduction in viscosity over a period of some hours. The initial viscosity of the solution was η_r 3·12 at 25·4° and after heating 5 min. at 80° the value fell to η_r 1·69, after which, on heating a further 10 hr. at 80°, the value obtained was η_r 1·35. Since, as will appear later, the solution of mucoid contained free gelatin, the slow continuous fall in viscosity which followed the first dramatic change may have been due to the degradation of this protein (cf. Ames, 1947).

Electrophoresis experiments

The experiments were carried out at 3·2° in the apparatus of Tiselius (1937) using the optical arrangement of Philpot as modified by Svensson (1939, 1946). In order to suppress boundary anomalies and to reduce the effects of viscosity the experiments were carried out with solutions containing high concentrations of salt and low concentrations of colloid. Phosphate-NaCl, glycine-NaCl and acetate-NaCl buffers were used, the solutions having ionic strength 0·02 with respect to the buffer ions and ionic strength 0·18 with respect to NaCl. The preparations of mucoid and protein were dialyzed against the appropriate buffer for 3–4 days until equilibrium was reached, the concentration of the colloid was then measured refractometrically and the solutions were adjusted to 0·25–0·5 % (w/v) by dilution with the buffer. The conductivities of the buffer and mucoid solution were measured at 0° and the mean of the two values was used in conjunction with the milliampere readings in order to determine a mean value for the potential gradient at the boundaries. Since conductivity was measured at 0° the values for mobility given in the tables refer to 0° rather than to the temperature at which the experiment was carried out. The pH of the buffer solutions was measured by means of a hydrogen electrode at 0°.

Electrophoresis of mucoid solutions. The results of a series of electrophoresis experiments carried out on the same batch of cartilage extract are given in Table 3. The extract was made at 60° in phosphate-NaCl buffer of pH 6·9 following the procedure already given, and, for the individual mobility determinations, samples of the extract were dialyzed against buffers of the desired pH.

Fig. 2 (*a*) shows the electrophoretic pattern obtained at pH 8·5 in phosphate-NaCl buffer (μ, phosphate, 0·02; μ, NaCl, 0·18). Over a range of pH values from 5·9 to 9·0 the patterns given were essentially similar. In the positive limb of the cell three boundaries were invariably present, and these were denoted *A*, *CP* and *B* in the order of their mobilities. In the negative limb, however, two boundaries only were visible, the mobilities corresponding to fractions *A* and *B*. The *A* peaks were sharp in both limbs, and comparison of the mobilities given for *A* in Table 3 with those found by Blix (1940) showed the *A* component to be chondroitin sulphate. This conclusion was afterwards confirmed by isolation of the component using the large apparatus of Tiselius (1938). The peaks due to substance *B* were sharp in the negative limb, but rather diffuse in the positive limb, and owing to the difficulty in measuring the position of the diffuse peak

the observed average values of mobility for *B* were subject to some variation. The observed values, however, were near to those recorded for the purified protein component of the mucoid (given in Table 4), and for this reason the peaks *B* were identified with the protein. The component *CP* gave a very sharp peak in the positive limb, but within the pH range indicated in Table 3 the boundary was absent in the negative limb. Although the sharpness of the peak facilitated mobility readings, its position varied rather considerably in different preparations and the mobility values recorded in Table 3 show some scatter. At pH 5·25 the peak *CP* showed in both limbs. At this acid reaction the preparations were usually rather opalescent and it was observed that after the current had been passed for some time opalescence was largely confined between the two *CP* boundaries.

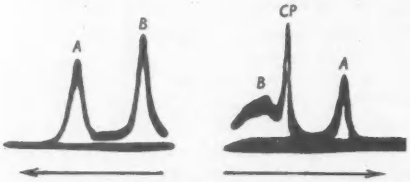

Fig. 2 *a*. Electrophoresis pattern of chondromucoid in phosphate-NaCl buffer at pH 8·5. The preparation was obtained by extracting cartilage powder with phosphate-NaCl solution (pH 6·9) at 60°. Left, negative, descending; right, positive, ascending.

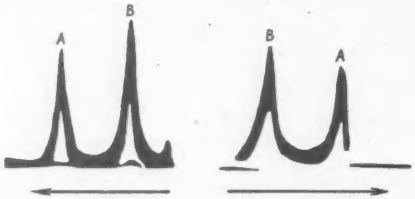

Fig. 2 *b*. Electrophoresis pattern of chondromucoid after heating at 100° for 30 min. in phosphate-NaCl solution of pH 6·88. Left, negative, descending; right, positive, ascending.

The reduction of viscosity on heating the cartilage extracts has already been reported, and Fig. 2 (*b*) shows the electrophoresis pattern obtained with a phosphate-NaCl extract (pH 6·88) of cartilage that had been heated at 100° for 30 min. in the same buffer in which the extract was made. In heated preparations the *CP* peak was invariably absent, and the two components *A* and *B* appeared with symmetrical peaks in both limbs. Table 3 shows the values for mobility of the two components in phosphate-NaCl buffers of pH 6·88 and 5·33. The time and temperature of heat treatment required to eliminate the component *CP* was found in all cases to be sufficient to complete the first rapid stage in the reaction leading to reduction of viscosity of the extracts, whether the heat treatment was carried out in phosphate-NaCl buffers of varying pH or in aqueous acetic acid solution. It thus appears probable that the component *CP* contributes significantly to the viscosity of the unheated solution. This conclusion is borne out by the extreme sharpness of the *CP* peak and also by the opalescent appearance associated with the complex, both of which observations indicate a particle of considerably greater size than either of the other two components in the system.

Table 3. *Electrophoretic pattern of cartilage extract*

Exp. no.	Buffer	pH (0°)	Mobilities (average from both limbs, $u \times 10^5$) Chondroitin sulphate	*CP* component	Protein
		Unheated extract			
25	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	5·25	−10·54	−5·64	−1·06
21	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	5·89	−10·90	−5·8*	−1·94
22	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	6·93	−11·35	−2·34*	−1·36
19	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	7·86	−11·68	−4·32*	−1·31
26	Glycine (μ, 0·02) +NaCl (μ, 0·18)	9·01	−11·76	−4·04*	−1·48
	Extract heated at 100° for 30 min. in phosphate-NaCl (pH 6·88) before dialysis against appropriate buffer				
35	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	6·88	−12·35	—	−1·12
36	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	5·33	−12·4	—	−1·22

* The boundary appeared in the positive limb of the cell, but was absent from the negative limb

Electrophoresis of the protein component. The protein component isolated by extraction of the dry mucoid with 90% (w/w) phenol solution was electrophoretically homogeneous over a pH range 3·6–7·0. The mobility readings in a series of buffers of total ionic strength 0·20 are recorded in Table 4. The boundaries in both limbs of the cell were sharp and symmetrical and the pH-mobility curve obtained by plotting the data in Table 4 was

Table 4. *Mobility of the protein isolated from chondromucoid*

Exp. no.	Buffer	pH	Mobilities ($u \times 10^5$)		
			u +ve	u −ve	Average
28	Glycine (μ, 0·02) +NaCl (μ, 0·18)	3·58	+2·29	+2·59	+2·44
30	Acetate (μ, 0·02) +NaCl (μ, 0·18)	4·29	+1·19	+1·19	+1·19
31	Acetate (μ, 0·02) +NaCl (μ, 0·18)	5·11	−0·31	−0·31	−0·31
32	Acetate (μ, 0·02) +NaCl (μ, 0·18)	5·53	−0·58	−0·47	−0·53
29	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	6·95	−0·85	−0·75	−0·80

generally of the shape found for gelatin (Moyer & Moyer, 1940). The isoelectric point of the protein in acetate-NaCl buffer (μ, acetate, 0·02; μ, NaCl, 0·18) was 4·86. This is close to the value (4·9) found by Moyer & Moyer for the isoelectric point of electrodialyzed commercial gelatin adsorbed on quartz or collodion particles, and since the solubility properties, gel-formation and analytical composition of the protein were similar to those of authentic gelatin, the protein must be regarded as having originated from collagen.

Electrophoresis of gelatin prepared from the collagenous residue remaining after extraction of the cartilage with alkaline $CaCl_2$ *solution.* The gel-forming protein prepared by heating the collagenous residue in an autoclave was similar in analytical composition to authentic gelatin, and showed a single sharp boundary in the electrophoresis apparatus. The mobility readings given by the protein fitted the mobility-pH curve obtained with the samples of gelatin isolated from the chondromucoid (Table 4). It thus appears that, apart from a small insoluble residue (comprising less than 6% of the intact tissue), the ground substance of cartilage is a structure built up from no more than two clearly recognizable chemical individuals, chondroitin sulphate and collagen; the process of successive degradation described in this work may therefore be looked upon as leading to the destruction of a collagen-chondroitin sulphate complex, and ultimately to the complete degradation of the collagen structure to gelatin and the liberation of chondroitin sulphate in a degraded form.

DISCUSSION

The work described in the experimental section shows that the intercellular matter of hyaline cartilage of beef septum nasi is composed almost exclusively of chondroitin sulphate and proteins of the collagen group, the relationship between these substances having been examined by degradation of the tissue material in a series of controlled steps.

The extreme sensitivity of collagen to increase in temperature led to the view that the collagen structure of cartilage would suffer modification at lower temperatures than does the chondroitin sulphate, and experiment showed that if the temperature of an aqueous suspension of powdered cartilage was raised to a point at which thermal contraction of the collagen would be expected to take place (60–70°) the treatment resulted in the release of a highly viscous, water-soluble mucoid containing chondroitin sulphate in a yield representing some 60% of the amount originally present in the tissue. The use of salt solutions, particularly those of the divalent alkaline earths, had the expected effect in reducing both the temperature of contraction of the collagen of rat-tail tendons and the temperature required to release chondromucoid from cartilage to approximately the same degree.

The analytical composition of the mucoid extracted from cartilage remained remarkably constant over a series of extraction experiments in which a number of solvents and salt solutions were used, provided the lowest effective temperature was employed, but the yield of chondroitin sulphate obtained was usually no more than 60–70% of that present in the tissue. If higher temperatures were used, the mucoid had a lower viscosity and contained an excess of protein. Extraction with 10% calcium chloride solution at pH 11 gave yields of 90–95% of the ester-bound sulphur originally present in the tissue, but here again, the viscosity of the extracted mucoid was low.

The water-soluble mucoid was examined in the electrophoresis apparatus of Tiselius over a considerable pH range and, in addition to the boundaries due to the protein and the acid polysaccharide, a third boundary, due to a complex between the two, was observed over the whole range covered by the experiments. From pH 9·0 to 5·8 the third boundary (designated *CP*) appeared in the positive limb only, but at pH 5·3 it showed its presence in both limbs and at this acid reaction the solution had a distinct opalescence which appeared to be confined between the two *CP* boundaries. The substance responsible for the *CP* boundary was heat labile and a further stage in the process of degradation was carried out by heating the mucoid at 80–100° for 10–30 min. when it no longer showed the presence of the third boundary in the electrophoresis apparatus and the protein and polysaccharide components migrated independently. The disappearance of the *CP* boundary on heating the mucoid was coincident with a considerable fall in the viscosity of the solution, and since the sharpness of the *CP* boundary indicated a particle of considerable length, it seems probable that the complex contributed significantly to the viscosity of the unheated solution. The failure of the component to appear as a distinct boundary in the negative limb at hydrogen-ion concentrations near neutrality may be due to the instability of the complex except in the presence of an excess of chondroitin sulphate. Such mass-action effects are common in antigen-antibody reactions and reveal their presence in zoning phenomena.

A similar position has been revealed by the work of Chargaff, Ziff & Moore (1941), who examined the electrophoretic behaviour of heparin when mixed with serum albumin or dialyzed plasma. A third component was observed (designated by these authors the *C component*), which had a mobility intermediate between that of serum albumin and the acid polysaccharide. In this case, however, the *C component* was more clearly discernible on the descending side than on the ascending side, which usually showed only a broadening of the albumin peak. Some preparations of heparin failed to produce the *C component*, but in this case the heparin peak appearing on the descending side was many times larger than on the ascending side. Chargaff and his colleagues also examined the effect of chondroitin sulphate on human plasma proteins, but no evidence of complex formation was obtained. However, a highly purified specimen of chondroitin sulphate was used and it may have been somewhat degraded in the process of purification.

Since the complex formation of heparin with serum albumin is of considerable importance in the anticoagulant activity of the former it was considered of interest to assay the activity of 'undegraded' chondroitin sulphate as an anticoagulant. However, it was found that both the mucoid itself and a preparation of chondroitin sulphate obtained from the mucoid by separation in the large apparatus of Tiselius (1938) had less than 2 % of the activity of a commercial preparation of heparin.

Complex formations between proteins and chondroitin sulphate at reactions more acid than the isoelectric point of the protein have been studied in some detail by Meyer *et al.* (1937), who show that the mucins produced are in fact true salts, and are formed in stoicheiometric proportions. The present demonstration of reversible complex formation at alkaline reactions, however, indicates association of a different type wherein the protein and the polysaccharide are both negatively charged. The character of the bonds involved in a union of the latter type is unknown, but provided the particle size of the components is sufficiently great, polar association is not excluded since, except under very strongly alkaline conditions, the more strongly basic centres of the protein molecule are still charged positively.

It is known that native collagen suffers a loss of ammonia on conversion into gelatin, and there is evidence that in its untreated condition collagen has an isoelectric point higher than that of gelatin prepared from it (cf. Ames, 1944). It is, therefore, probable that conditions in the intact tissue are different from those obtaining in the extracted mucoid. However, since complex formation occurs between chondroitin sulphate and protein in the mucoid at hydrogen-ion concentrations within the physiological range, it seems reasonable to presume that it also occurs in the intact tissue. This assumption provides an answer to a point that has frequently been debated. The early workers, in particular Mörner (1889), held that chondroitin sulphate occurred in the tissue as an alkali metal salt, while Meyer *et al.* (1937) were of the view that the tissue contains a salt between chondroitin sulphate and protein. It now appears that the position lies between the two extremes, and that the strongly acid sulphate groups are most probably held in combination with some of the basic residues of the protein, the net charge of the complex being adjusted mainly by a competition between alkali metal ions and hydrogen ions for carboxylic acid residues in both protein and polysaccharide.

It has not proved possible to secure the liberation of mucoid from the tissue by any treatment sufficiently mild to avoid modifying collagen, and it is therefore clear that the mucoid, particularly as regards its protein moiety, should be looked upon as an artifact. However, the circumstance that chondroitin sulphate retains its capacity of complex formation in the water-soluble system resulting from degradation of the tissue affords some insight into the structure of the tissue itself. If our knowledge

of the chemical behaviour of the polysaccharide is combined with the results of histological investigations the structure may be visualized as a network of collagen fibrils, in some places organized into parallel bundles to form microscopic fibres and in others relatively disorganized and heavily cross-linked by association with chondroitin sulphate. The extraction experiments also suggest that the protein of the mucoid springs from the 'disordered' collagen of the cementing substance rather than from the ordered fibres.

This view of the structure of the tissue is supported by the recent observations of Cohen (1942), who showed that many proteins of plant origin having a molecular weight greater than 10^6 were precipitated by heparin, chondroitin sulphate and hyaluronic acid, and in some cases gave rise to extremely long particles often of paracrystalline form. Large concentrations of the colloidal anion were required to effect the precipitation, but only small quantities of the polysaccharides were carried down by the crystalline precipitates.

These facts invite speculation as to the part played by chondroitin sulphate in the orientation of collagen in developing connective tissue. In tissue cultures and healing wounds the fibres arise from fibroblasts which migrate into the medium or the exudate from the wound, the fibres afterwards becoming oriented into fibre bundles, finally adopting the characteristic form of true collagenous tissue. It may be that the typical structure of collagen in cartilage and connective tissue generally is the result of a process similar in character to the production of the paracrystals observed by Cohen (1942) in which chondroitin sulphate, acting as a multivalent anion, cements together the protein molecules to form fibrous macromolecules and eventually fibre bundles. It is noteworthy that even the most regular white connective tissue, that in tendon, always contains appreciable amounts of mucoid.

SUMMARY

1. The intercellular substance of bovine nasal cartilage is composed almost exclusively of two major components—collagen and chondroitin sulphate.

2. A soluble mucoid may be extracted from dried cartilage powder after a short heat treatment with water at 60–70°.

3. The temperature required to liberate mucoid is reduced in the presence of inorganic salts, alkalis and formamide, and the conditions necessary to secure extraction in good yield are generally those which give rise to thermal contraction of collagen.

4. The mucoid contains chondroitin sulphate and a protein derived from the degradation of collagen. Prepared under the lowest effective temperature conditions, the mucoid behaves in the electrophoresis apparatus of Tiselius as an equilibrium mixture of chondroitin sulphate, protein and a complex formed between the two.

5. The association of chondroitin sulphate with protein in the mucoid occurs over the range pH 5–9 within which both the protein and the acid polysaccharide are negatively charged. The complex formation is of a different type from the salt formation known to occur below pH 4·85.

6. On further heating, the mucoid loses its capacity to form complexes at pH 5–9 and suffers a rapid reduction in viscosity.

7. The part played by such complex formation in the intact tissue is discussed and it is suggested that chondroitin sulphate has an important role in the organization of collagen in developing connective tissue.

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The Influence of Bactericidal Agents and of the Absence of L-Ascorbic Acid on the Accumulation of Hydroxyphenyl Compounds in the Large Intestine of Guinea Pigs after the Consumption of High Doses of L-Tyrosine

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In a previous communication (Painter & Zilva, 1947) it was shown that very much larger quantities of hydroxyphenyl compounds* were found in the large intestine, mainly in the caecum, 3 or 5 hr. after the ingestion of high doses of L-tyrosine when guinea pigs were depleted of L-ascorbic acid, than when they received cabbage *ad lib.* with the basal diet. It was further observed that a similar accumulation in the large intestine could be found when sulphaguanidine was administered to guinea pigs subsisting on the basal diet with cabbage *ad lib.* In this paper experimental evidence is given which affords an explanation of this phenomenon, and observations have been made which are of fundamental theoretical interest and have possibilities of therapeutic application.

EXPERIMENTAL

Technique. For details of experimental animals and diet, see Penney & Zilva (1946). For methods of collection of urine, preparation of tissues for analysis, determination of total hydroxyphenyl compounds ('tyrosine'), and L-tyrosine, see Painter & Zilva (1947).

The samples of sulphaguanidine, succinylsulphathiazole and phthalylsulphathiazole used were commercial medicinal products.

Administration of the bactericidal agents. In the most intensive experiments of long duration (8–15 days), the compounds were administered as aqueous suspensions in quantities varying from 0·1 to 0·3 g., the doses being adjusted from day to day so as to prevent a loss in weight of the animals. In the case of the experiments of shorter duration (3 days) daily graded doses of 0·05, 0·1, 0·2 and 0·3 g. of sulphaguanidine were administered to the respective groups. Experiments have proved that guinea pigs on a diet containing cabbage *ad lib.*, even after dosage with sulphaguanidine for 8 days, were fully saturated in respect of L-ascorbic acid.

* Hydroxyphenyl compounds are usually determined by methods which are based on Millon's reaction. A negative reaction is then taken to indicate rupture of the benzene ring, although in reality it only indicates the disappearance of the phenolic group. For the sake of greater accuracy we abstain in this communication from referring to the rupture of the benzene ring in cases in which the Millon's reaction is absent.

RESULTS

The 'tyrosine' content of the large intestine of guinea pigs after the administration of L-tyrosine under different dietetic conditions.

Table 1 summarizes the results with sixteen groups, each of 12 guinea pigs. All the animals were killed 3 hr. after the administration of 0·5 g. of L-tyrosine and the contents of the large intestine, comprising the caecum, colon and rectum, were immediately analyzed for hydroxyphenyl compounds by Folin & Ciocalteu's method (1927). The values thus obtained are calculated as 'tyrosine'. No significant quantities of these compounds were found in the faeces passed during the 3 hr. between the administration of the L-tyrosine and slaughter. References in the text to animals depleted for a certain time signify the time which elapsed since the removal of the cabbage, previously administered *ad lib.*, from the basal scorbutic diet. The statistical treatment of the data was carried out as described by Snedecor (1937) for the determination of the *t* value.

The mean value for 'tyrosine' obtained with the animals receiving cabbage *ad lib.* in addition to the basal scorbutic diet during the pre-experimental period (group 1) was very low compared with that obtained with the animals which were depleted for 14 days (group 6). Group 1 also has a wide variation of the individual values. No other group, with the possible exception of group 16, showed this characteristic. This is particularly evident when the high ratio of the standard deviation to the mean is compared with similar ratios in the other groups. This observation is in accordance with expectation, if it be assumed that the quantity of 'tyrosine' found in the large intestine is controlled by the quantity of cabbage present there when the administered L-tyrosine reaches the large intestine in considerable quantities. The amount of the cabbage present would in such a case depend on the quantity eaten and on the time when it was consumed and this would vary with the individual animal. In all the other groups the conditions were more under the control of the experimenter and thus the fluctuations were not so marked.

Table 1. *The 'tyrosine' content of the large intestine of guinea pigs 3 hr. after the administration of 0·5 g. L-tyrosine to animals previously maintained under various dietetic conditions*

Group no.	Addition to basal diet	Days	Treatment on day of L-tyrosine dosing	Mean 'tyrosine' content (expressed as mg. L-tyrosine)	S.D.	$\frac{\text{S.D.}}{\text{Mean}} \times 100$
1	Cabbage *ad lib.* in the pre-experimental period	0	—	24	29	121
2	Nil	1	—	94	39	42
3	Nil	3	—	134	51	38
4	Nil	5	—	104	49	47
5	Nil	8	—	106	30	28
6	Nil	14	—	159	46	29
7	25 mg. L-ascorbic acid daily	6	—	65	32	49
8	Nil	1	25 mg. L-ascorbic acid with the L-tyrosine	86	41	48
9	Nil	1	100 mg. L-ascorbic acid with the L-tyrosine	58	31	53
10	Nil	14	100 mg. L-ascorbic acid with the L-tyrosine	88	46	52
11	Nil	6	100 mg. L-ascorbic acid with the L-tyrosine	85	39	46
12	Nil	14	25 mg. L-ascorbic acid with the L-tyrosine	108	53	49
13	Nil	14	100 mg. L-ascorbic acid 1 hr. before, with, and 1 hr. after the L-tyrosine	76	47	62
14	Nil	14	Cabbage *ad lib.* immediately after the L-tyrosine	92	35	38
15	Nil	14	100 mg. L-ascorbic acid with the L-tyrosine and cabbage *ad lib.* immediately after	98	24	25
16	Nil	14	Cabbage *ad lib.* 2 hr. before the L-tyrosine, 100 mg. L-ascorbic acid with the L-tyrosine and cabbage *ad lib.* after	29	26	90

That the disappearance of the 'tyrosine' from the large intestine depends on the constant presence of cabbage is seen from the fact that after the removal of the cabbage from the diet for 24 hr. (group 2), the quantity of 'tyrosine' present in the large intestine became significantly higher than in group 1 ($t = 4{\cdot}98$), but it still remained significantly lower than that found after 14 days' depletion (group 6, $t = 3{\cdot}73$). Unfortunately, an attempt to find out how soon after the removal of the cabbage from the diet the maximum intestinal content of 'tyrosine' might be reached was not completely successful as experiments carried out 3, 5 and 8 days after the removal of the cabbage from the diet (groups 3–5) yielded values which were not always significantly different among themselves, and sometimes not even so when compared with the mean obtained after 14 days' depletion (cf. groups 3 and 6, $t = 1{\cdot}26$). This is possibly due to the great individual variation in the time during which the unaltered cabbage remains in the lower reaches of the intestinal tract.

The first attempt to ascertain whether the degradation of the phenolic group of the L-tyrosine in the large intestine was connected with the vitamin C content of the cabbage was made by administering daily 25 mg. of synthetic L-ascorbic acid in solution for 6 days to guinea pigs maintained on the basal scorbutic diet, the last dose of the vitamin being given with the L-tyrosine. It was found (group 7) that the mean of the contents of this group was of the same order as that of group 2 (animals depleted for

24 hr.), but was, nevertheless, significantly higher than that of group 1 which received cabbage *ad lib.* ($t = 3{\cdot}29$). The essential point, however, is that the means of groups 3–6 which were depleted for 3, 5, 8 and 14 days respectively ($t = 3{\cdot}97$, $2{\cdot}31$, $3{\cdot}24$ and $5{\cdot}81$ respectively) were markedly higher. In other words, the presence of L-ascorbic acid *per se* is capable of producing a speedier disappearance of the phenolic group of the 'tyrosine' in the intestine. It is of interest to note that after 1 day's depletion the 'tyrosine' values could not be lowered by the administration of 25 or 100 mg. of L-ascorbic acid with the L-tyrosine dose (*vide* groups 8 and 9). Yet after 6 or 14 days' depletion the 'tyrosine' values could be lowered to that obtained after 1 day's depletion by the administration with the L-tyrosine of 25, 100 and 300 mg. (in three successive doses of 100 mg.) of L-ascorbic acid (*vide* groups 10–13).

Various attempts to attain the low 'tyrosine' values observed in the case of guinea pigs on a diet containing cabbage *ad lib.* were made. This was accomplished only when cabbage was offered *ad lib.* to guinea pigs 2 hr. before the administration of 0·5 g. of L-tyrosine with 100 mg. of L-ascorbic acid and subsequent cabbage *ad lib.* (group 16). On the other hand, the administration to guinea pigs depleted for 14 days of (*a*) three successive doses of 100 mg. of L-ascorbic acid, one before, one with and one after the dose of L-tyrosine (group 13), (*b*) of cabbage *ad lib.* with the L-tyrosine (group 14) and (*c*) of 100 mg. of L-ascorbic acid in addition to cabbage *ad lib.* with the dose of L-tyrosine (group 15), only lowered the large intestine 'tyrosine' values to values approximating that of the mean of the group of animals depleted for 1 day (group 2). It will be noted that in group 16 the individual values are as widely distributed as in group 1, probably for the same reason.

The evidence in this series of experiments definitely supports the view that the consumption of vitamin C favours the disappearance of the phenolic group of L-tyrosine or of the hydroxyphenyl degradation products of this compound from the intestine. The higher efficiency of cabbage as an agent in this process of degradation as compared with L-ascorbic acid in solution cannot be fully explained at present. Two possible explanations are worth considering. One is that during the transit of the cabbage in the alkaline section of the intestine the vitamin is more protected, and that in the large intestine there is a gradual release of the L-ascorbic acid. In consequence, the total quantity of the vitamin is more efficiently utilized in the reaction in question than when the L-ascorbic acid is in solution, and is more accessible for destruction through other agencies. Alternatively, the cabbage, apart from its physical condition, may contain another substance which conduces to the breakdown of L-tyrosine in the intestine. We incline to the former view.

The influence of bactericidal agents on the degradation of L-tyrosine in the intestines of guinea pigs

The results of the above experiments strongly indicated the possibility that the accumulation of 'tyrosine' in the large intestine of the depleted guinea pigs was the consequence either of a change in the intestinal flora which was caused by the absence of L-ascorbic acid or of a change in the capacity of some of these organisms to metabolize the phenolic group of L-tyrosine and its degradation products. The obvious approach to the elucidation of the problem was, therefore, to study the influence of bactericidal agents known to modify the bacterial flora of the intestine. The action of sulphaguanidine, succinylsulphathiazole and phthalylsulphathiazole was accordingly investigated with this point in view. In each case it was found that these agents when administered *per os* to animals receiving cabbage *ad lib.* produced, as in the case of the depleted animals, an accumulation of 'tyrosine' in the large intestine, mainly in the caecum. A detailed and comparative study of guinea pigs receiving cabbage *ad lib.* and sulphaguanidine as the bactericidal agent on the one hand, and depleted guinea pigs on the other, has, however, revealed some differences between the changes brought about by the presence of the bactericidal agent and the absence of L-ascorbic acid from the diet. These differences, as will be seen later, did not, however, militate against the hypothesis that the absence of L-ascorbic acid and the presence of the bactericidal agent were capable of modifying the intestinal flora of the guinea pig so as to interfere with their degradation of the L-tyrosine present there.

The results of this group of experiments are summarized in Tables 2 and 3. The outstanding fact which emerges from Table 2 is that 3 hr. after the administration of L-tyrosine there is a great accumulation of 'tyrosine' in the large intestine of the depleted guinea pigs and of the animals which received cabbage *ad lib.* and sulphaguanidine, when compared with the content found in the case of the animals receiving only cabbage *ad lib.* Even a daily dose of 0·1 g. of sulphaguanidine given for 3 days previous to the administration of L-tyrosine already produced this effect. The data in Table 2 disclose a further point which, apart from its general interest, has a bearing on the present problem. It will be seen that the concentration and contents of the hydroxyphenyl compounds in the tissues of the entire intestinal tract in the groups of the depleted animals and of the animals receiving cabbage *ad lib.* were much higher than in the case of the groups which were dosed with sulphaguanidine. In the

Table 2. *The distribution of 'tyrosine' in the body and urine of guinea pigs maintained on diets containing cabbage* ad lib. *with and without sulphaguanidine, and that of guinea pigs on a synthetic diet 3 hr. after the administration*

Table 2. *The distribution of 'tyrosine' in the body and urine of guinea pigs maintained on diets containing cabbage* ad lib. *with and without sulphaguanidine, and that of guinea pigs on a scorbutic diet 3 hr. after the administration of 0·5 g.* L-*tyrosine*

		Mixed diet containing cabbage										Scorbutic diet	
Daily dose of sulphaguanidine ...		None		0·1–0·3 g.		0·1 g.		0·2 g.		0·3 g.		None	
No. of days		0		12–15		3		3		3		14	
No. of animals		5		7		6		6		6		6	
Found in tissues		Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)	Content (mg.)	Conc. (mg./100 g.)
Stomach tissue:	Mean	1·9	56	Trace	Trace	0·7	22	0·7	25	0·7	25	2·0	67·3
	S.D.	0·8				0·33		0·56		0·73		0·8	
Stomach contents:	Mean	6·8	—	3·8	—	20·0	—	15·1	—	13·8	—	7·0	—
	S.D.	5·4		4·1		28·3		14·8		19·3		7·7	
Small intestine tissue:	Mean	3·7	32	Trace	Trace	1·2	10	0·9	8	Trace	Trace	5·4	40
	S.D.	3·1				1·18		0·5				1·8	
Small intestine contents:	Mean	3·0	—	Trace	—	Trace	—	2·6	—	Trace	—	2·8	—
	S.D.	1·7						2·2				0·8	
Large intestine tissue:	Mean	3·3	37	Trace	Trace	1·0	8	1·1	9	0·6	5·5	4·1	39·2
	S.D.	3·3				0·84		0·64		0·52		2·0	
Large intestine contents:	Mean	21·1	—	150	—	103	—	60·5	—	130	—	149	—
	S.D.	33·2		38·1		74·9		18·9		64·4		60·3	
Blood:	Mean	7·7	38·5	Trace	Trace	2·1	10	2·6	13	1·7	9	11·6	58
	S.D.	5·2				1·6		1·6		1·2		3·9	
Liver:	Mean	2·7	26	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	5·2	41·6
	S.D.	2·3										1·2	
Kidney:	Mean	1·7	69	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	2·0	75·7
	S.D.	2·0										0·9	
Muscle:	Mean	6·5	12·9	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	29·7	57
	S.D.	1·7										16·5	
Urine:	Mean	11·1	—	7·0	—	0·6	—	6·0	—	2·7	—	36·1	—
	S.D.	12·05		3·54		0·56		3·58		2·4		21·6	

Table 3. *Summary of the distribution of 'tyrosine' (expressed as mg.* L-*tyrosine) in the body and urine of guinea pigs maintained on diets containing cabbage* ad lib. *with and without sulphaguanidine, and that of guinea pigs on a scorbutic diet, after the administration of 0·5 g.* L-*tyrosine*

	Mixed diet containing cabbage									Scorbutic diet	
Daily dose of sulphaguanidine ...	None		0·1–0·3 g.		0·1 g.		0·2 g.	0·3 g.	0·05 g.	None	
No. of days	0		12–15	8	3		3	3	3	14	
Time after L-tyrosine dose (hr.) ...	3	24	3	24	3	24	3	3	24	3	24
No. of animals	5	6	7	12	6	6	6	6	6	6	4
Contents of intestinal tract + faeces	30·9	Trace	154	21·3	123	Not done	78·2	143·8	Not done	159	Trace
Tissues	27·0	Trace	Trace	Not done	5·0	Not done	5·3	3·0	Not done	60	Trace
Urine	11·1	16·2	7·0	24·7	0·6	18·7	4·8	2·7	22·6	36	205
Total	69·0	16·2	161·0	46·0	128·6	18·7	88·3	149·5	22·6	255	205
Percentage not accounted for	86	97	68	91	74	96	82	70	95	49	59

latter animals, although the size of the dose and the time during which it was administered did not influence the content of 'tyrosine' in the large intestine to an appreciable extent, it did, nevertheless, influence the content and concentration of the hydroxyphenyl compounds in the walls of the intestinal tract. In the animals dosed intensively (0·1–0·3 g. sulphaguanidine for 12–15 days) only traces of 'tyrosine' could be found in the intestinal tissues, but low concentrations, bearing a roughly inverse proportion to the dose, could be observed in the animals treated less drastically with the bactericidal agent (0·1, 0·2 and 0·3 g. sulphaguanidine for 3 days). The latter 'tyrosine' values were, however, considerably lower than those found in the case of the animals receiving no sulphaguanidine. The difference in the 'tyrosine' content and concentration between the animals dosed with the sulphaguanidine and the other guinea pigs is even more marked in the case of the blood, liver, kidney and muscle. All these differences could be explained by an impaired passage of the 'tyrosine' into the blood stream brought about by the consumption of the bactericidal agent.

This view was strengthened by further evidence. In Table 3 are given the 'tyrosine' contents of (*a*) the intestinal tract and faeces, (*b*) the tissues, and (*c*) the urine of all the animals 3 hr. (summarized from Table 2) and 24 hr. after the administration of the dose of L-tyrosine. It is seen that while the greater part of the 'tyrosine' in the tissues and large intestine found 3 hr. after the administration of the L-tyrosine to the depleted guinea pigs was eliminated by the kidney during the following 21 hr. (169 out of 219 mg.), only about one eighth was accounted for in the urine of the sulphaguanidine-treated guinea pigs. This fact taken in conjunction with the observation that the tissues of these animals, as mentioned above, contained little or no 'tyrosine' when compared with the tissues of the guinea pigs which did not receive sulphaguanidine, supports the view that the consumption of sulphaguanidine greatly impaired the passage of the 'tyrosine' into the blood stream. That some of these compounds did pass into the circulation is seen from the fact that the mean of the 'tyrosine' content of the urine values of the animals receiving sulphaguanidine for 12–15 days was $24{\cdot}7 \pm 15{\cdot}6$ mg. (Table 3). This value was significantly higher than the mean we have previously obtained from a control group of 12 guinea pigs not receiving L-tyrosine ($6{\cdot}3 \pm 4{\cdot}1$ mg.; $t = 3{\cdot}95$). Furthermore, we have found that, by the prolonged administration of L-tyrosine to guinea pigs receiving sulphaguanidine, 'tyrosine' values reaching 60 mg./day could be attained. Therefore, although some of the 'tyrosine' did enter the system, the best part of it seems to have disappeared from the gut, but at a very much slower rate than in the case of guinea pigs receiving cabbage *ad lib.* without sulphaguanidine. In the case of these animals only 57·9 mg. of 'tyrosine' out of the 500 mg. of L-tyrosine received was present in the large intestine and in the tissues 3 hr. after the administration of L-tyrosine against 154 mg. present, entirely in the large intestine, in the case of guinea pigs which received 100–300 mg. of sulphaguanidine daily for 12–15 days. After a further 21 hr. not a trace was found in the intestines and tissues in the former group of animals, whilst in the latter 21·3 mg. 'tyrosine' was still present in the intestinal contents and in the faeces. The evidence indicates that the bactericidal agent had significantly diminished the intestinal bacterial population which is capable of attacking the phenolic group in L-tyrosine, and in consequence the phenolic group of the L-tyrosine or of the hydroxyphenyl degradation products was destroyed to a smaller extent during the first few hours than in the case of the animals on the same diet which did not receive sulphaguanidine.

DISCUSSION

The evidence of this investigation leaves little doubt that the accumulation of 'tyrosine' in the large intestine 3 hr. after the administration of L-tyrosine in guinea pigs on a diet not containing cabbage is due to the absence of L-ascorbic acid *per se* and that the accumulation of 'tyrosine' in the gut of the depleted animals is primarily responsible for the incomplete breakdown of the phenolic group in some of the animals receiving a massive dose of L-tyrosine. The reason why the administration of cabbage is more effective than L-ascorbic acid in obviating the accumulation of 'tyrosine' in the large intestine is not quite clear, but in all probability it is due to the greater protection of the L-ascorbic acid when the cabbage is passing through the alkaline region of the small intestine and to the gradual release of the vitamin from the plant tissue. It was further seen that a similar accumulation of 'tyrosine', but without the appearance in the urine of an excess of hydroxyphenyl compounds, can be produced by the administration to guinea pigs of bactericidal agents such as sulphaguanidine, succinylsulphathiazole and phthalylsulphathiazole even when the animals consume cabbage *ad lib.* In order to obtain a clear understanding of the problem it is necessary to correlate the present results with those described by us in a previous communication (Painter & Zilva, 1947).

In the first investigation we demonstrated that when a high enough dose of L-tyrosine was injected in depleted guinea pigs intraperitoneally as glycyl-L-tyrosine, i.e. when the concentration of L-tyrosine in the blood was raised to a certain critical level, there was an increase in the amount of the total hydroxyphenyl compounds eliminated by the kidney and *p*-hydroxyphenylpyruvic acid was excreted in the

urine at the same time.* The phenolic group in a part of the L-tyrosine was, therefore, not broken down in the system. Very much higher doses of glycyl-L-tyrosine had to be injected into animals receiving cabbage to bring about this incomplete degradation of the L-tyrosine, evidently because of the much higher concentration of L-ascorbic acid in the blood of these animals. It was, therefore, not unwarranted to assume that a critical concentration of 'tyrosine' in the blood, similar to that obtained by injection of high doses of glycyl-L-tyrosine, could also be attained by permeation from the large intestine of depleted guinea pigs. The accumulation in the lower reaches of the intestinal tract caused by the absence of L-ascorbic acid from the diet of these animals must, therefore, be considered primarily responsible for the partial degradation of the phenolic group in the large dose of L-tyrosine. The fate of the 'tyrosine' accumulated in the large intestine of the guinea pigs which received cabbage and sulphaguanidine was different. It will be recalled that the intestinal tissues, blood, liver, kidney and muscle did not contain any appreciable quantities of 'tyrosine', and that only about one eighth of the accumulated 'tyrosine' was accounted for in the urine. This strongly suggested that little of these hydroxyphenyl compounds entered the blood stream and that the highest concentration thus reached was below the critical concentration necessary to produce an abnormal degradation of the compounds in question attained in the depleted guinea pigs. Consequently, no *p*-hydroxyphenylpyruvic acid was formed under these conditions, not even when L-tyrosine was administered on several successive days. Since, however, the greater part of the accumulated 'tyrosine' disappeared within the next 21 hr. (*vide* Table 3) it may be assumed that these compounds have been slowly destroyed in the intestine, as compared with the animals which received cabbage without sulphaguanidine, especially as some 'tyrosine' was, under these circumstances, still present in the intestinal contents and in the faeces. We are, therefore, faced with the following outstanding facts: (*a*) A quick destruction of 'tyrosine' in the case of the guinea pigs receiving L-ascorbic acid, since the amount present in the large intestine after 3 hr. was small, and although this entered freely into the blood stream containing 'saturated values' of L-ascorbic acid it could not reach the critical concentration above which the system was incapable of metabolizing these compounds normally. Only 3 % of the 0·5 g. of L-tyrosine was accounted for after 24 hr., the remainder having been destroyed. (*b*) A much slower destruction of the 'tyrosine' with a resultant accumulation in the large intestine after 3 hr. in the case of the guinea pigs receiving cabbage and sulphaguanidine. The bulk of this was slowly destroyed in the lower reaches of the intestine during the following 21 hr. In this case 4–9 % of the consumed L-tyrosine was accounted for (Table 3), most of it being destroyed in the intestine. (*c*) A slow destruction of the 'tyrosine' in the depleted animals with an accumulation in the large intestine. In this case, however, the hydroxyphenyl compounds enter the circulation freely, and the concentration in the blood is such that in the presence of little ascorbic acid the phenolic group cannot be degraded in all the 'tyrosine', and at the same time some phenolic compounds are produced which are evidently intermediate in the degradation of the L-tyrosine. After 24 hr. 41 % of the L-tyrosine was accounted for in the urine as hydroxyphenyl compounds (Table 3).

If the possibility of a very rapid passage into the blood stream is excluded, the evidence appears to justify the assumption that the L-tyrosine in the intestine of the guinea pig is normally quickly destroyed by intestinal organisms and that the rate of this destruction can be slowed down to a considerable extent either by administering bactericidal agents or by withholding L-ascorbic acid from the diet. In the former case the explanation is fairly clear; the bactericidal agent diminishes the population of the intestinal organisms including those destroying L-tyrosine. In the latter case other possibilities may have to be considered. The presence of L-ascorbic acid may favour organisms, capable of attacking the phenolic group, which are suppressed in the absence of the vitamin. The vitamin may even act as a necessary link in a chain of reactions concerned in this degradation. The literature on the subject does not throw much light on the problem. The action of various organisms on L-ascorbic acid has been investigated by several workers (Stepp & Schröder, 1935; Kendall & Chinn, 1938; Esselen & Fuller, 1939; Young & James, 1942; Young & Rettger, 1943). Certain strains of these organisms under the requisite conditions can oxidize L-ascorbic acid, mostly beyond the reversible stage, but some, such as *Escherichia coli*, which is actually capable of reducing dehydro-L-ascorbic acid, can act in a protective capacity. Fortunately, according to Crecelius & Rettger (1943), the normal flora of the guinea pig intestine is relatively simple, consisting mainly (about 80 %) of a non-sporulating rod form of *Lactobacillus*. The remainder of the flora is composed of yeasts and of soil and air bacteria. The investigation of the action of these organisms on phenolic

* In our description of the method of determination of *p*-hydroxyphenylpyruvic acid (Painter & Zilva, 1947) we stated inadvertently that the solution of the compound in 2·5 % metaphosphoric acid used for the calibration curves was allowed to remain 30 min. after being adjusted to pH 9·0. This should have read that the solution of *p*-hydroxyphenylpyruvic acid was adjusted to pH 9·0, allowed to remain for 30 min., after which metaphosphoric acid was added so that the final concentration was 2·5 %.

amino-acids in the presence and in the absence of L-ascorbic acid may supply information not only of fundamental theoretical interest but also lead to therapeutic applications. The observation that some bactericidal agents are capable of modifying under these abnormal conditions the passage of 'tyrosine' into the blood stream is also of fundamental theoretical and practical interest. As was seen, this impairment of the passage was only partial, since some of the hydroxyphenyl compounds have been shown to enter the blood stream. It may also be noted that the guinea pigs receiving the bactericidal agent were found to be saturated in respect of L-ascorbic acid. This problem requires further investigation.

SUMMARY

1. Three hours after the administration of 0·5 g. of L-tyrosine more hydroxyphenyl compounds were found in the large intestine, particularly in the caecum, of guinea pigs which were depleted of L-ascorbic acid and in that of guinea pigs receiving sulphaguanidine, succinylsulphathiazole or phthalylsulphathiazole with cabbage *ad lib.* than in guinea pigs receiving vitamin C in the form of cabbage or L-ascorbic acid in solution without the bactericidal agents.

2. A number of experiments are described in which the influence of the mode of administration and of the quantity of the added L-ascorbic acid was investigated, and it is concluded that L-ascorbic acid as present in cabbage is more effective than as the synthetic compound in solution.

3. A great part of the hydroxyphenyl compounds present in the large intestine of the depleted guinea pigs 3 hr. after the administration of the L-tyrosine was found in the urine of the animals 21 hr. later. Only a small portion of these accumulated compounds was present in the urine of the guinea pigs receiving cabbage *ad lib.* and sulphaguanidine.

4. The concentrations of hydroxyphenyl compounds in the walls of the intestine, the blood, the liver, the kidney and the muscle were much higher in the depleted animals and in the animals receiving cabbage *ad lib.* without sulphaguanidine than in the corresponding tissues of animals receiving the bactericidal agent.

5. Excluding a very rapid passage into the blood stream, it may be assumed that the lower content of hydroxyphenyl compounds in the large intestine of the animals receiving cabbage *ad lib.* or synthetic L-ascorbic acid in solution without sulphaguanidine is due to the influence of the intestinal bacterial flora which is modified by either the absence of L-ascorbic acid from or the presence of bactericidal agents in the diet of the guinea pigs. In the first case, owing to the free passage into the circulation, the concentration in the blood reaches levels which are too high for these hydroxyphenyl compounds to be metabolized normally. In the second case the impaired passage into the blood stream brought about by the consumption of the bactericidal agents prevented the production of such a critical concentration of hydroxyphenyl compounds in the circulation, and the greater part of these compounds was destroyed slowly during passage along the large intestine.

6. The fundamental and practical potentialities of these observations are stressed.

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Pipette for Use with the Van Slyke-Neill Manometric Apparatus

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In the use of the Van Slyke-Neill manometric apparatus, the accurate measurement of reagents maintained free from dissolved air is much facilitated by use of the special pipette illustrated (Fig.1). This pipette allows 50 ml. reagent to be kept out of contact with air for an indefinite period and for any required quantity to be accurately measured into the apparatus without any possibility of coming into contact with air.

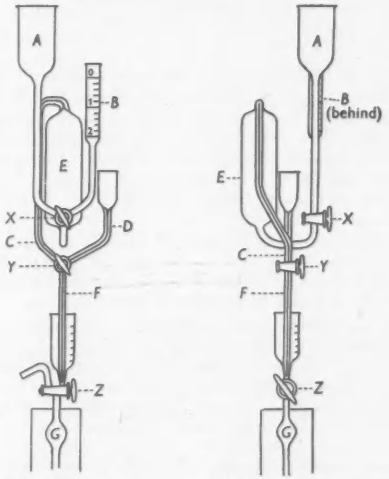

Fig. 1. Pipette for use with the Van Slyke-Neill manometric apparatus.

Construction

The pipette is built round two 3-way stopcocks *X* and *Y*, with branches at 120° and with an angle of 120° in the bore so that any two branches may be connected. Stopcock *Y* should have capillary bore connexions. Bulb *E* and cup *A* should have about 50 ml. capacity. *B* is a calibrated tube; for most purposes a capacity of 2 ml. $\times \frac{1}{20}$ is convenient since it is unusual to measure greater volumes of reagent into the chamber of the Van Slyke apparatus, but, if required, the capacity of this tube may be greater. The bottom of cup *A* should be approximately level with the zero mark on *B* and the top of *E* should be approximately level with the lowest graduation on *B*. Tubes *D*, *F* and *C* should be of capillary bore throughout. In those models of Van Slyke's apparatus with a standard ground socket at the top of the reaction chamber, *F* should be fitted with a corresponding standard cone; otherwise the tip of *F* is fitted with a piece of rubber as usual in the use of Ostwald pipettes. The pipette should be built as compactly as possible, otherwise the weight of mercury in *A* and *E* will be liable to fracture the tubing. If it is so built the apparatus is reasonably sturdy, but a stand with three clips to support the pipette when not in use is an advantage, especially if several pipettes are required.

Operation

Filling pipette. (*a*) *Filling with mercury.* To fill the pipette open *X* to connect *A* to *E*, and *Y* to connect *C* to *F*. Pour mercury in through *A* until *E* is full and mercury flows from *F*. Close *X* and *Y* and add about 15 ml. more mercury to *A*. Open *X* to connect *A* to *B* until mercury reaches the zero mark in *B*, then close *X*. Pour mercury into *D*, open *Y* to connect *D* to *F* until mercury flows from *F*.

(*b*) *Introduction of reagent.* Free the reagent from air in the Van Slyke apparatus in the usual manner. Place a small quantity of mercury in the cup above chamber *G*, insert the tip of *F*. Raise the levelling bulb of the Van Slyke apparatus to the upper ring, open *Z* to connect *G* to *F*, open *Y* to connect *F* to *C* and *X* to connect *E* to *A*. The solution will flow into *E* displacing mercury into *A*. When all the reagent is in the pipette or the mercury is almost entirely driven from *E*, close *X*, *Y* and *Z*, remove the pipette, open *Y* to connect *D* to *F* so that mercury fills the bore of *F*.

Addition of measured amount of reagent to the Van Slyke apparatus. Place the tip of *F* in the cup above chamber *G* of the Van Slyke apparatus (the cup should contain a few drops of mercury). Open *X* to connect *B* to *E* and *Y* to connect *C* to *F*. Turn *Z* slowly until the mercury in *B* indicates that the required volume of reagent has entered chamber *G*. Close *X*, open *Y* to connect *D* to *F* until mercury flows into *G*; close *Y* and *Z*.

To set the pipette for use again, open *X* to connect *A* and *B* and allow mercury to rise to the zero mark in *B*.

It is not usually necessary to empty the pipette. If a different reagent is to be put into it, the aqueous contents may be removed by placing the pipette on the Van Slyke apparatus, opening *X* to connect *A* to *E* and *Y* to connect *C* to *F* and drawing them down into *G*. The fluid is then expelled from *G* to waste, and *G* refilled with water which is passed up into the pipette, this process being repeated until the pipette is clean. Should it become necessary to empty mercury out of the pipette this is most conveniently done by holding the pipette face downwards over a suitable wide container and withdrawing the plug of cock *X*.

SUMMARY

A special pipette is described for use with the Van Slyke manometric apparatus. It allows storage of reagents out of contact with air for indefinite periods and measurement of any required quantity of reagent into the manometric apparatus without its coming into contact with air.

Synthesis of Glutamic Acid in Animal Tissues*

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In earlier experiments (Krebs & Henseleit, 1932) it was noted that slices of rat liver, suspended in a saline medium containing ammonium salts, often removed more ammonia from the medium than could be accounted for by the formation of urea. The present work arose from a study of the reactions other than the synthesis of urea, which cause a disappearance of ammonia in liver tissue.

EXPERIMENTAL

Analytical methods. Urea was determined manometrically with urease (Krebs & Henseleit, 1932; Krebs, 1942); NH_3 was steam-distilled *in vacuo* according to Parnas and Heller, as described by Krebs (1935), and measured photoelectrically after nesslerization. Amino N was determined with Van Slyke's (1929) manometric nitrous acid method, after deproteinization with trichloroacetic acid and removal of NH_3. Glutamic acid was determined according to Gale (1945) with the decarboxylase of *Clostridium welchii*, strain S.R. 12. Succinic and α-ketoglutaric acids were determined by the succinic dehydrogenase method (Krebs & Eggleston, 1948*a*), acetoacetate with aniline citrate (Edson, 1935), and oxaloacetate with $Al_2(SO_4)_3$ (Krebs & Eggleston, 1945).

Tissue preparations. Homogenates were made in the glass apparatus of Potter & Elvehjem (1936), and in a similar instrument made from stainless steel. The medium used for the dilution of the tissue was 'phosphate saline without Ca' (Krebs & Eggleston, 1940) or phosphate bicarbonate saline without Ca. Lower rates of reaction were found when Ca was present in physiological concentrations. A medium simulating the ionic composition of liver cells (containing 6 ml. of 0·1 M-$CaCl_2$, 18 ml. of 0·1 M-$MgCl_2$, 15 ml. of 0·1 M-K_2HPO_4, 10 ml. of 0·1 M-KH_2PO_4, 10 ml. of 0·155 M-KCl and 41 ml. of 0·155 M-NaCl) gave rates of about 25% and, when Ca was omitted, of about 90% of those in 'phosphate saline without Ca'. The homogenates were used as freshly as possible. On storage their activity, as measured by their capacity to form glutamic acid, fell fairly rapidly, e.g. by *c.* 50% when kept in the refrigerator for 24 hr.

Slices were suspended in phosphate saline or phosphate bicarbonate saline.

Adenosine triphosphate (*ATP*). Ba-ATP (64·7 mg.) was dissolved in 3·3 ml. of 0·1 N-HCl. Ba was precipitated by the addition of 0·1 ml. of M-Na_2SO_4 and the solution was neutralized with 0·15 ml. of 2 N-NaOH. Water was added to 5 ml. The precipitate was removed by filtration or centrifugation. The solution contained 0·02 M-ATP.

General. The material was incubated in conical Warburg vessels. The substrates were placed in the side arm and added to the tissue preparation at zero experimental time, usually when the manometers were attached to the water bath. The temperature was 40°. All substrate concentrations mentioned in the tables refer to the final concentrations in the medium. In anaerobic experiments yellow P was placed in the centre well. Quantities of metabolites are expressed in μl. and rates of metabolic processes in the usual *Q* values. When homogenates were used the dry weight for calculating the *Q* values was assumed to be 30% of the fresh weight in the case of liver and 20% in the case of brain cortex, kidney cortex or muscle.

To stop the metabolic processes in homogenates after a specified time the flasks were detached from their manometers and 1 ml. of 2 N-HCl/4 ml. suspension was added as quickly as possible. If the analyses could not be carried out immediately the solutions were stored in the refrigerator. Of the acidified suspension usually 1 ml. was used for the determination of glutamic acid, 1 ml. for NH_3, 3 ml. for succinic acid. If a larger amount of material was required for further analyses, duplicate Warburg cups were set up and the contents were combined after completion of the incubation.

* Preliminary account, *Nature, Lond.*, **159**, 808 (1947).

Homogenates often contained significant amounts of NH_3, glutamic acid and other metabolites. These initial concentrations were determined in a sample acidified with 2N-HCl (0·25 vol.) at the start of the experiment.

RESULTS

Excess of ammonia consumption over urea formation. Examples showing the quantities of ammonia removed by rat-liver slices in relation to urea formation are shown in Table 1. The amount which cannot be accounted for by the urea formation varied with the presence of other substrates and was of the same order of magnitude as those converted into urea. In these experiments no ornithine or citrulline was added. In the presence of these substances the excess of ammonia consumption over urea formation was usually small.

rat liver was only a small fraction, usually less than 10%, of the rate observed in slices, unless ornithine or citrulline was added. For example, Q_{urea} was 7·1 in slices and 0·25 in homogenized rat liver (substrate 0·01 M-ammonium chloride). Similar results have been reported by Cohen & Hayano (1946). Homogenates, however, utilize ammonia if suitable substrates are present (Table 2). Of the substrates tested α-ketoglutarate had the greatest effect on the utilization of ammonia; citrate was slightly less effective, pyruvate and oxaloacetate still less. No appreciable effects were found with fumarate, lactate, glucose and acetoacetate. With α-ketoglutarate the rate observed in this experiment was of the same order as in slices.

Effect of tissue dilution. In the preceding experiments 1 part of the tissue was diluted with medium to about 7 parts of suspension. Dilution to 15 parts

Table 1. *Urea formation and ammonia consumption in rat-liver slices*

(Slices suspended in 4 ml. phosphate saline. Incubation 1 hr.)

Substrates	Tissue (mg.)	Urea (μl.)	Q_{urea}	NH_3 left (μl.)	NH_3 removed (μl.)	Q_{NH_3}	Amount of NH_3 (μl.) Accounted for by urea	Amount of NH_3 (μl.) Not accounted for by urea
None	12·60	13	1·03	Nil	Nil	0	—	—
NH_4Cl (0·005 M)	13·30	53	3·98	250	−180	−13·52	80	100
+ L-lactate (0·02 M)	8·65	35·5	4·10	358	− 72	− 8·32	45	27
+ pyruvate (0·02 M)	8·50	34	4·00	328	−102	−12·00	42	60
+ oxaloacetate (0·02 M)	13·80	57	4·13	250	−180	−13·04	88	92
+ glucose (0·02 M)	11·80	57	4·84	285	−145	−12·29	88	57

Table 2. *Removal of ammonia in rat-liver homogenate*

(One part of liver homogenized with 4 parts of phosphate bicarbonate saline; 3 ml. diluted with 1 ml. of substrate solution or saline; 5% CO_2; 95% O_2; 40°; incubation 20 min.; concentration of other substrates 0·01 M; NH_4Cl added, 430 μl.; NH_4Cl found in suspension at start, 63 μl.)

Substrate added (in addition to NH_4Cl)	NH_3 found (μl.)	Change in NH_3 (μl.)	Q_{NH_3}
α-Ketoglutarate	116	−377	−6·3
Citrate	266	−227	−3·8
Pyruvate	318	−175	−2·9
L-Lactate	482	− 11	−0·2
Acetoacetate	469	− 24	−0·4
Fumarate	486	− 7	−0·1
Glucose	512	+ 19	+0·3
Oxaloacetate	395	− 98	−1·6
None	512	+ 19	+0·3

Ammonia utilization in liver homogenates. It was thought advantageous for the present purpose to work under conditions in which the urea synthesis was suppressed whilst other reactions involving the utilization of ammonia still proceeded. This object was largely realized by the use of homogenized liver. After homogenization the rate of urea synthesis in decreased the initial rate of ammonia removal in proportion to the tissue concentration; in other words Q_{NH_3} remained constant. In the subsequent experiments a final tissue concentration of 1 part of tissue in 9 parts of medium was used unless otherwise stated.

Effect of oxygen and of saline media. Rat-liver homogenates, in the presence of α-ketoglutarate, also remove ammonia anaerobically. The data in Table 3 show that the anaerobic rate can even be higher, by about 15%, than the aerobic rate. The presence of bicarbonate and carbon dioxide, which is of importance for the formation of urea, did not affect the rate of the reaction responsible for the removal of ammonia in the present experiment.

Since anaerobic conditions reduce the number of reactions that occur in tissue preparations, and thus simplify the interpretation of results, the majority of the following experiments were carried out anaerobically.

Formation of glutamic acid. Determinations of amino N in the solutions of the experiment recorded in Table 2 showed an increase in amino N approximately equivalent to the loss of ammonia. The fact that α-ketoglutarate was the most effective substrate in removing ammonia and forming amino N

Table 3. *Aerobic and anaerobic removal of* NH_3 *in the presence of α-ketoglutarate in rat-liver homogenate*

(One part of liver homogenized with 4·5 parts of saline; each cup contained 3 ml. of homogenate and 1 ml. of additional solution (substrate or saline). Incubation 30 min. The homogenate contained 90 μl. NH_3 per cup from internal sources at the start of the experiment. The data refer to 4 ml. of suspension.)

Medium	Phosphate saline				Phosphate bicarbonate saline			
Gas	O_2		N_2		5% CO_2 in O_2		5% CO_2 in N_2	
NH_4Cl added (μl.)	0	914	0	914	0	914	0	914
α-Ketoglutarate added (μl.)	0	1792	0	1792	0	1792	0	1792
NH_3 found after incubation (μl.)	71	264	124	242	102	322	150	276
Change in NH_3 (μl.)	−19	−650	+34	−678	+12	−592	+60	−638
Same corrected for suspension without added NH_4Cl		−631		−712		−604		−698
Q_{NH_3}		−7·7		−8·7		−7·4		−8·5

suggested that the reaction under investigation was the reductive amination of α-ketoglutarate, and the solutions were therefore tested for glutamic acid by Gale's (1945) method. It will be seen from Table 4 that the quantities of ammonia removed and

Table 4. *Formation of glutamic acid in rat-liver homogenate*

(One part of liver, 9·5 parts of phosphate saline; N_2; 40°; 40 min. incubation. The corrected values were obtained by deducting the values found in the absence of substrate.)

Substrates added (final concentration)	None	α-Ketoglutarate (0·01 M) NH_4Cl (0·01 M)
NH_3 change (μl.) (observed)	+45	−318
(corrected)	—	−363
Glutamic acid (μl.) (formed)	+40	+395
(corrected)	—	+355

glutamic acid formed agree within the limits of error if corrections are made for changes in the tissue which occur without the addition of substrates. Other examples showing this agreement are found in Tables 5 and 6; in some experiments the formation of glutamic acid was rather smaller than the removal of ammonia.

Formation of succinic acid. The reductive amination of α-ketoglutarate is necessarily coupled with an equivalent oxidative process. Previous observations in this laboratory (Krebs & Cohen, 1939) suggested that the oxidative decarboxylation of α-ketoglutarate might be the equivalent reaction. The experimental data recorded in Table 5 show

Table 5. *Anaerobic formation of succinic acid*

(Conditions as in Table 4.)

Substrates added	None	α-Keto-glutarate (0·01 M) NH_4Cl (0·01 M)
NH_3 change (μl.) (observed)	+58	−322
(corrected)	—	−380
Glutamic acid found (μl.) (observed)	+84	+415
(corrected)	—	+331
Succinic acid found (μl.) (observed)	+18	+394
(corrected)	—	+376

that this is essentially true: the yields of glutamic and succinic acids and the amounts of ammonia used in this experiment are roughly in accordance with the equation

$$2\ \alpha\text{-Ketoglutarate} + NH_3 \rightarrow \text{glutamic acid} + \text{succinate} + CO_2. \quad (1)$$

Table 6. *Comparison of changes in* NH_3, *glutamic acid and succinic acid in liver homogenates*

(Final tissue concentration 10%; α-ketoglutarate and NH_4Cl 0·01 M; 'blank' refers to tissue incubated without substrate.)

Species	Period of incubation (min.)		NH_3 (μl.)	Glutamic acid (μl.)	Succinic acid (μl.)
Guinea pig	40	Blank	+ 42	+ 95	+ 35
		With substrates	−287	+375	+222
		Data corrected for blank	−329	+280	+187
Guinea pig	40	Blank	+ 37	+ 93	+ 20
		With substrates	−336	+430	+398
		Data corrected for blank	−373	+337	+378
Rat	60	Blank	+106	+ 65	+ 15
		With substrates	−236	+305	+205
		Data corrected for blank	−342	+240	+190
Rat	20	Blank	+ 21	+ 17	0
		With substrates	−353	+253	136
		Data corrected for blank	−374	+236	136

In other experiments, however, the yield of succinate was low and the disappearance of ammonia high (Table 6). The former observation was taken to indicate that other oxidative reactions can be coupled with the synthesis of glutamic acid; the latter suggests that additional reactions causing the removal of ammonia occur in liver homogenates.

Effects of various metabolites on the synthesis of glutamic acid. In order to find the other reactions which might be coupled with the synthesis of glutamic acid, various substrates were added together with α-ketoglutarate and ammonium chloride under anaerobic conditions. No definite effects on the yield and rate of formation of glutamic acid were found with glucose, glycogen, glucose-1-phosphate, acetate, crotonate, acetoacetate, glycerol, α-glycerophosphate, ethanol, stearate and choline. Three sets of substances (Table 7) ((*a*) citrate and the two related tricarboxylic acids, (*b*) β-hydroxybutyrate, (*c*) fumarate and L-malate) increased the rate of glutamic acid synthesis as well as the final yield, which is 50% of the added α-ketoglutarate if reaction (1) is the only mechanism leading to glutamic acid. Major inhibitions of both glutamic acid synthesis and ammonia consumption were found with L-lactate and hexosediphosphate (Table 8). Acetate and butyrate caused slight inhibitions in some experiments. L-Alanine increased, and pyruvate decreased, the yield of glutamic acid owing to transamination.

Examples showing the effects of citrate, β-hydroxybutyrate and fumarate in guinea-pig liver are given in Table 7. In this experiment, as in all similar ones, citrate had the greatest effect. It increased the removal of ammonia and the formation of glutamic acid almost equally without appreciably affecting the formation of succinate. The sum of the α-ketoglutarate, succinate and glutamate found exceeded by 440 μl. the amount of α-ketoglutarate added. This can be explained by the assumption that citrate was converted into α-ketoglutarate under the conditions of the experiment. The yields and the calculations concerning the oxido-reduction balance are in agreement with the view that the reductive amination of α-ketoglutarate (found 782 μl.) was coupled with the following two oxidative reactions:

Citrate → α-ketoglutarate (found 440 μl.).
α-Ketoglutarate → succinate (found 350 μl.).

The effect of β-hydroxybutyrate was much smaller than that of citrate but was also definite. The yield of glutamate, which in the absence of added substrate does not exceed 50% of the α-ketoglutarate (as expected from equation 1), reached 59%, while the yield of succinate showed a slight decrease. The presence of acetoacetate in the solution indicates that the additional oxidative reaction coupled with the glutamate formation was the oxidation of β-hydroxybutyrate to acetoacetate.

In the case of fumarate the yield of glutamate also exceeded 50%; it was 64%. The fact that oxaloacetate appeared indicates that the reaction fumarate → oxaloacetate was in this case one of the oxidative equivalents of the glutamate formation. That the amount of oxaloacetate found was re-

Table 7. *Effect of citrate, β-hydroxybutyrate and fumarate on the synthesis of glutamic acid in guinea-pig liver homogenate*

(General conditions as in Table 6; incubation 40 min.; substrate concentrations all 0·01 M except DL-β-hydroxybutyrate which was 0·02 M. Controls showed that in the absence of α-ketoglutarate and NH_4Cl citrate yielded no α-ketoglutarate, β-hydroxybutyrate no acetoacetate and fumarate no oxaloacetate.)

Substrates added ...	None	α-Keto-glutarate, NH_4Cl	α-Keto-glutarate, NH_4Cl, citrate	α-Keto-glutarate, NH_4Cl, DL-β-hydroxy-butyrate	α-Keto-glutarate, NH_4Cl, fumarate
NH_3 (μl.) change	+37	−336	−754	−496	−612
Corrected		−373	−791	−533	−649
Glutamic acid formed (μl.)	+93	+430	+875	+600	+663
Corrected		+337	+782	+507	+570
Succinate formed (μl.)	+20	+398	+370	+380	+438
Corrected		+378	+350	+360	+418
Acetoacetate formed (μl.)	0	0	0	+210	0
Oxaloacetate formed (μl.)	0	0	0	0	+ 38
α-Ketoglutarate found (μl.)	0	104	204	49	0
α-Ketoglutarate added (μl.)	0	896	896	896	896
Sum of α-ketoglutarate, succinate and glutamic acid recovered (corrected)		819	1336	916	988
Oxido-reduction balance					
Reductions:					
Formation of glutamic acid		337	782	507	570
Oxidations:					
Formation of succinate		378	350	360	418
Formation of acetoacetate or oxaloacetate			790	570	456
Formation of α-ketoglutarate			440	210	38

latively small is not unexpected because of its rapid secondary reactions. Some oxaloacetate must have reacted with glutamate by transamination; hence the amount of glutamate found was smaller than that of the ammonia removed. Another fraction of oxaloacetate probably reacted to form the tricarboxylic acids, α-ketoglutarate and malate (Krebs, Eggleston, Kleinzeller & Smyth, 1940); hence the sum of α-ketoglutarate and succinate exceeded the amount of α-ketoglutarate added. In all experiments on guinea-pig liver the effect of fumarate was of a similar magnitude; in rat liver the effect was smaller, but also marked.

The effects of citrate, β-hydroxybutyrate and fumarate suggest that the reductive amination of α-ketoglutarate can be coupled with four different oxidative reactions (*a*) α-ketoglutarate → succinate, (*b*) isocitrate → α-ketoglutarate, (*c*) β-hydroxybutyrate → acetoacetate, (*d*) fumarate (or L-malate) → oxaloacetate.

Examples of the inhibitory effect of lactate, hexosediphosphate, acetate and butyrate are given in Table 8. The inhibition of glutamic acid formation by 0·01 M-L-lactate in guinea-pig liver was 73 %. The inhibition of the ammonia uptake was of the same order. No explanation for the mechanism of this inhibition can be offered.

Effect of adenosinetriphosphate. If the experimental periods were short (less than 10 min.) and the tissue concentrations in the homogenate high (10 % or more), addition of ATP had little or no effect on the rate of glutamic acid synthesis. In longer experiments with more dilute suspensions, ATP had definite effects in that it delayed the decline in the rate. These effects were larger if sodium fluoride, which inhibits the hydrolysis of ATP, was also present (see Table 9). The effects may be taken to indicate that ATP is a component of the system responsible for the synthesis of glutamic acid.

Effect of substrate concentration. As Table 9 indicates, the rate of glutamic acid synthesis in homogenates falls quickly with time. In further experiments, designed to measure the initial rates, a short incubation period of 10 min. was therefore chosen. When the concentration of α-ketoglutarate was varied between 0·005 and 0·04 M, the highest rates were found at 0·02 M. Variations of the concentration of ammonium chloride between 0·05 and 0·02 M, with a concentration of α-ketoglutarate of 0·02 M, had no effect on the rates.

Rate of glutamic acid synthesis in homogenates and slices of various tissues. Table 10 compares the rates of the synthesis of glutamic acid in homogenates with those in slices of liver and kidney. In the case

Table 8. *Inhibition of glutamic acid synthesis by lactate and other substances in liver homogenates*

(Concentration of α-ketoglutarate and NH_4Cl, 0·01 M; N_2; data refer to 4 ml.)

Species	Period of incubation (min.)	Additional substrates	Glutamic acid found (μl.)	NH_3 removed (μl.)
Rat	20	—	237	375
		L-Lactate, 0·02 M	68	86
		Na acetate, 0·01 M	180	165
Guinea pig	15	—	201	—
		L-Lactate, 0·01 M	55	—
		Hexosediphosphate, 0·01 M	33	—
		Na butyrate, 0·01 M	150	—

Table 9. *Effects of adenosine triphosphate and* NaF *on the rate of glutamic acid synthesis in rat-liver homogenates*

(Medium: phosphate saline; 40°; N_2; 0·02 M-α-ketoglutarate; 0·01 M-NH_4Cl. The initial values for glutamic acid in the homogenates have been deducted.)

No.	Tissue concentration (%)	Additional substrates added	Glutamic acid formed (μl.) 10 min.	20 min.
1	10	None	255	263
		ATP, 0·001 M	277	295
		NaF, 0·02 M	223	273
		ATP, 0·001 M; NaF, 0·02 M	281	368
			15 min.	30 min.
2	5	None	99	126
		ATP, 0·001 M	141	166
		NaF, 0·02 M	78	101
		ATP, 0·001 M; NaF, 0·02 M	125	206

Table 10. *Comparison of the rates of glutamic acid synthesis in homogenates and in slices*

(Data for homogenates and slices given in the same horizontal line refer to the same tissue except where an asterisk is added; 0·02 M-α-ketoglutarate; 0·01 M-NH_4Cl. Incubation period for homogenates 10 min.; N_2.)

Tissue	Homogenate $Q_{glutamic\ acid}$	Slices: Period of incubation (min.)	Slices: $Q_{glutamic\ acid}$
Liver, rat	12·8	20	1·0
Liver, rat	12·3	20	2·0*
Liver, rat	9·3	30	4·8*
Liver, rat	10·5	30	4·1*
Liver, guinea pig	9·8	20	4·5
Liver, sheep	3·3	15	1·0
Liver, pigeon	15·5	30	2·7*
Liver, pigeon	17·6	—	—
Kidney cortex, rat	7·1	30	4·7*
Kidney cortex, rat	2·0	30	2·7*
Kidney cortex, rat	5·2	—	—
Kidney cortex, sheep	3·3	15	3·2
Kidney cortex, sheep	4·2	15	2·6

of homogenates the values given are corrected for the initial amount of glutamic acid, and with slices for the amount of glutamic acid found on incubation without addition of substrates. It will be seen that in rat-liver homogenates $Q_{glutamic\ acid}$ was between 9·3 and 12·8, against 1·0–4·8 in slices. For the livers of the guinea pig, sheep and pigeon the rates were likewise much greater in homogenates. On the other hand, the differences between sliced and homogenized material were small in kidney cortex of the rat and sheep.

In slices the rate of glutamic acid synthesis increased with time, in contrast to the fall that occurred in homogenates. Examples are given in Table 11. The accelerating effect of citrate already reported for homogenates was also found in slices. In the liver and kidney of sheep and rats citrate approximately doubled the rate of synthesis; in homogenates the increase was usually about 50 %. The highest rates of glutamic acid synthesis in the presence of citrate were, in terms of $Q_{glutamic\ acid}$, 27 in pigeon liver and 20 in rat liver.

Table 11. *Time course of glutamic acid synthesis in sliced tissue*

(0·02 M-α-ketoglutarate; 0·01 M-NH_4Cl; N_2.)

Tissue	Time of incubation	$Q_{glutamic\ acid}$
Liver, sheep	First 15 min.	1·0
	Second 15 min.	2·1
Kidney, sheep	First 15 min.	3·2
	Second 15 min.	6·5

In brain, minced heart muscle and pigeon breast muscle, small but measurable quantities of glutamic acid were always found on incubation with α-ketoglutarate and ammonium chloride. Addition of citrate usually caused a considerable increase (Table 12). Negligible rates ($Q_{glutamic\ acid} < 1$) were found in sheep spleen and guinea-pig lung.

Synthesis of glutamic acid in relation to the removal of ammonia in sliced liver. The question arises whether the synthesis of glutamic acid is one of the reactions other than urea synthesis causing a disappearance of ammonia in liver slices. To test this, liver slices were shaken aerobically under conditions similar to those stated in Table 1, and the amounts of ammonia removed, and of urea and glutamic acid formed, were measured (Table 13). Only a small proportion of the ammonia removed which was not accounted for by the formation of urea was recovered as glutamic acid. The proportion of the latter was largest when citrate was the substrate, but even in this case amounted to only 23 % of the missing nitrogen. There was no formation of glutamine under

Table 12. *Rate of glutamic acid synthesis in various tissues*

(0·02 M-α-ketoglutarate; 0·01 M-NH_4Cl; 0·01 M-citrate; N_2.)

Tissue	Substrates added	Period of incubation (min.)	$Q_{glutamic\ acid}$
Brain cortex, sliced, sheep	α-Ketoglutarate, NH_4Cl	30	0·8
	α-Ketoglutarate, NH_4Cl, citrate	30	1·5
Brain cortex, sliced, guinea pig	α-Ketoglutarate, NH_4Cl	30	0·9
	α-Ketoglutarate, NH_4Cl, citrate	30	2·3
Brain cortex, sliced, guinea pig	α-Ketoglutarate, NH_4Cl	30	1·3
	α-Ketoglutarate, NH_4Cl, citrate	30	4·8
Brain cortex, sliced, pigeon	α-Ketoglutarate, NH_4Cl	30	1·0
	α-Ketoglutarate, NH_4Cl, citrate	30	1·4
Brain, minced, sheep	α-Ketoglutarate, NH_4Cl	20	0·4
	α-Ketoglutarate, NH_4Cl, citrate	20	0·4
Heart, minced, sheep	α-Ketoglutarate, NH_4Cl	20	1·4
	α-Ketoglutarate, NH_4Cl, citrate	20	2·6
Striated muscle, minced, pigeon	α-Ketoglutarate, NH_4Cl	20	1·5
	α-Ketoglutarate, NH_4Cl, citrate	20	1·8

Table 13. *Formation of glutamic acid in rat-liver slices in the presence of* NH_4Cl *and other substrates*

(Phosphate saline; O_2. The data refer to 30 mg. (dry) tissue suspended in 4 ml. of saline, and are corrected for amounts formed by tissue without added substrate; concentration of NH_4Cl, 0·005 M; of other substrates, 0·01 M.)

Exp. no.	Substrates added	Period of incubation (min.)	NH_3 removed (μl.)	NH_3 removed not accounted for by urea (μl.)	Glutamic acid found (μl.)
1	NH_4Cl	60	167	161	27
2	NH_4Cl	90	96	71	0
	NH_4Cl, citrate	90	51	43	10
	NH_4Cl, glucose	90	9	9	0
	NH_4Cl, fumarate	90	0	0	0

Table 14. *Removal of glutamic acid by rat-liver slices*

(Phosphate saline; O_2. The data refer to 40 mg. (dry) tissue suspended in 4 ml. phosphate saline, and are corrected for amounts formed by tissue without added substrate.)

Exp. no.	Substrates added	Period of incubation (min.)	Glutamic acid removed (μl.)	Urea + NH_3 formed (as NH_3) (μl.)
1	L-Glutamate, 0·01 M	60	172	6
	L-Glutamate, 0·01 M; NH_4Cl, 0·005 M	60	161	51
2	L-Glutamate, 0·005 M	90	93	19
	L-Glutamate, 0·005 M; NH_4Cl, 0·005 M	90	93	40
3	L-Glutamate, 0·005 M	90	140	65
4	L-Glutamate, 0·005 M	90	74	34

the conditions of the experiments. For the interpretation of this result it is of importance, however, that liver tissue is capable of removing considerable quantities of glutamic acid without forming equivalent quantities of urea or ammonia, as is shown in Table 14. Accumulation in the slices did not account for an appreciable amount of the glutamic acid removed from the medium. The non-appearance of glutamic acid is therefore no proof of its non-formation. Glutamic acid may be used up in secondary reactions, and it is therefore possible that the synthesis of glutamic acid accounts for a greater proportion of the missing nitrogen than the data in Table 13 suggest.

DISCUSSION

The occurrence of a synthesis of glutamic acid in animal tissues was first recorded by Euler, Adler, Günther & Das (1938) and Dewan (1938). The main new contribution of the present work is the measurement of the rate of this reaction under varying conditions, and the observation that the reductive amination of α-ketoglutarate can be coupled with the reaction L-malate → oxaloacetate, as an alternative to the three reactions α-ketoglutarate → succinate + CO_2 (Krebs & Cohen, 1939), isocitrate → α-ketoglutarate + CO_2 (Adler, Euler, Günther & Plass, 1939), and β-hydroxybutyrate → acetoacetate (Dewan, 1938).

Physiological significance of the synthesis of glutamic acid. There are many indications that glutamic acid has a special function in the metabolism of various cells, though so far little information is available on this point. The rapid synthesis of glutamic acid in liver may serve partly to supply the requirements of this acid for the whole body, but the major part probably reacts by 'transimination', according to Cohen & Hayano (1946), and is thus a link in urea synthesis. The rates of transimination and of glutamic acid synthesis are of the same order and sufficient to account for the formation of urea, the highest value for Q_{urea} observed in rat liver being about 20 (Krebs & Henseleit, 1932) and for $Q_{glutamic\ acid}$ about 20. The stage citrulline + NH_3 → arginine of the ornithine cycle can therefore be resolved into the two stages:

α-Ketoglutarate + NH_3 → glutamic acid. (1)

Glutamic acid + citrulline → arginine. (2)

It has always been obvious that the synthesis of urea through the ornithine cycle, being an endergonic process, must be coupled with exergonic reactions, but no information on the nature of the exergonic reactions was previously available. It is now clear that the oxidative reactions which are coupled with the amination of α-ketoglutarate are components of the system of exergonic reactions supplying the energy for the synthesis of urea.

Several facts indicate that reaction (2) is a complex process. It requires the presence of oxygen (Borsook & Dubnoff, 1941) and is inhibited by low concentrations (0·005 M) of malonate (Cohen & Hayano, 1946). The malonate inhibition is abolished

by fumarate (Fahrländer, Favarger, Nielsen & Leuthardt, 1947). This effect of fumarate is a catalytic one; under suitable conditions one molecule of fumarate (or L-malate) can cause the formation of many more than five molecules of arginine (Krebs & Eggleston, 1948*b*). Ratner (1947) suggests that reaction (2) consists of two stages:

Glutamic acid + oxaloacetate → aspartic acid + α-ketoglutarate.

Aspartic acid + citrulline → L-malate + arginine.

Whilst the effect of fumarate on the malonate inhibition would be explained by this scheme it is difficult to account for the following four observations on rat liver homogenates (Krebs & Eggleston, 1948*b*):

(1) Aspartate and citrulline yield arginine very slowly if the conditions are anaerobic, the rate being less than 5% of that observed aerobically with glutamate and citrulline.

(2) Aerobically, glutamate and citrulline react about five times faster to yield arginine than aspartate + citrulline.

(3) Aspartate removes the malonate inhibition of reaction (2) less effectively than fumarate, the restoration being 50% with 0·005M-aspartate against 100% with 0·005M-fumarate.

(4) Malonate (0·005M) completely abolishes the interaction between aspartate and citrulline in oxygen; glutamate or α-ketoglutarate restores the capacity of liver homogenates to form arginine.

Differences between sliced and homogenized material. It would not be surprising to find that the rate of metabolic processes taking place in slices of animal tissues is greatly reduced, even to zero, by homogenization of the tissue, but it is somewhat unexpected to observe the reverse, i.e. a rapid rate in homogenates against an almost negligible rate in slices. The first to report such a phenomenon were Cohen & Hayano (1946) working on transimination in rat liver. The present observations on the reductive amination of α-ketoglutarate in sliced and homogenized rat material are in general similar to those on transimination. In both cases there is no major difference in sliced and homogenized kidney, whilst in rat liver the rates in homogenates are about three to fifteen times greater than in slices. To explain the relatively low rate of reaction in liver slices one may assume that either the enzyme systems concerned are largely inactive in the intact cell, owing to the presence of intracellular inhibitors, or that permeability barriers prevent the access of the substrates to the enzymes. Whatever the explanation of the phenomenon it is clear that major differences exist in the behaviour of kidney and liver cells.

SUMMARY

1. A search has been made for reactions, other than urea synthesis, causing a disappearance of added ammonia in isolated liver preparations. Homogenization of liver was found to abolish, under certain conditions, the conversion of ammonia into urea, whilst ammonia was still removed when pyruvate, oxaloacetate, α-ketoglutarate, or citrate were added. The main reaction responsible for the removal of ammonia in homogenates, on addition of the above substrates, was found to be the synthesis of glutamic acid.

2. The synthesis of glutamic acid in the presence of α-ketoglutarate and ammonia occurred both anaerobically and aerobically, the anaerobic rates being somewhat higher than the aerobic rates.

3. The reductive amination of α-ketoglutarate by liver can be coupled with the following four oxidative reactions: α-ketoglutarate → succinate + CO_2 (Krebs & Cohen, 1939); isocitrate → α-ketoglutarate + CO_2 (Adler *et al.* 1939); β-hydroxybutyrate → acetoacetate (Dewan, 1938); L-malate → oxaloacetate (not recorded previously).

4. L-Lactate and hexosediphosphate, and to a less extent acetate and butyrate, inhibited the formation of glutamic acid from α-ketoglutarate and ammonia.

5. Adenosine triphosphate delayed the fall in the rate of glutamic acid synthesis which occurred in homogenates when the experimental period exceeded 10 min.

6. The rate of glutamic acid synthesis in liver homogenates was three to fifteen times greater than in liver slices. In kidney cortex the differences in the rates observed in homogenized and sliced material were small.

7. The highest recorded rates of glutamic acid synthesis, in terms of $Q_{\text{glutamic acid}}$, were 20 for rat liver, 27 for pigeon liver, 8 for kidney cortex (sheep, rat), 5 for brain cortex, 3 for heart muscle, 2 for pigeon breast muscle, <1 for spleen and lung.

8. Glutamic acid was removed by liver slices without forming ammonia or urea, an indication of the occurrence of unknown reactions in which glutamic acid is utilized.

9. The synthesis of glutamic acid is responsible for a part, but probably not for the whole, of the ammonia which is removed by liver slices and not accounted for by urea formation.

10. The physiological significance of the synthesis of glutamic acid is discussed. One of the reactions for which glutamic acid is required in liver tissue is presumably the conversion of citrulline into arginine in the course of the synthesis of urea (Cohen & Hayano, 1946).

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The Determination of Quinine Degradation Product in Blood, and its Absorption in the Chick

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In a previous publication (Marshall, 1945*a*) we reported that quinine degradation product (QDP) showed no antimalarial action against *Plasmodium gallinaceum* in young chicks, when given orally in amounts equivalent to an active dose of quinine. Subsequently, Kelsey, Oldham, Cantrell & Geiling (1946) reported that, when given intravenously, the compound showed one third to one fifth the activity of quinine against chick malaria, and less than one twentieth that of quinine against *P. lophurae* when given orally to ducks. Pointing out that QDP was less toxic than quinine, thus making its therapeutic index nearly equal to that of quinine, these workers disagreed with our view that the metabolism of quinine results in an inherently less efficient drug. They suggested that the reduction of both antimalarial activity and toxicity might be due to a decreased rate of absorption in the host.

In order to test this theory, we decided to compare the concentrations of quinine and QDP attained in the red cells of chicks following oral doses of the compounds, and this necessitated the designing of a method of determination of small quantities of QDP. The fluorescence of QDP in ultraviolet light was too feeble for the determination of very small amounts, though it has been used to determine large concentrations (Knox, 1946). We found, however, that the bromothymol blue method (Marshall & Rogers, 1945) was almost as sensitive with QDP as with quinine.

The degradation product, which is prepared by incubating quinine with rabbit liver suspension, was shown by Mead & Koepfli (1944) to be quinine carbostyril, i.e. quinine with a hydroxyl group substituted in position 2 of the quinoline ring. This confers phenolic properties on the compound, making it unextractable from sodium hydroxide solution, thus providing a ready method for the separation of QDP from quinine. The degradation product can be extracted from ammoniacal solution (Kelsey, Geiling, Oldham & Dearborn, 1944). We have used the basic principles of this method, but emulsification problems made quantitative extraction difficult. However, by modifying the technique suitably, and carefully controlling the shaking operations, a method giving satisfactory recoveries of small amounts of QDP was produced.

METHODS

Quinine degradation product. This was prepared by incubating quinine with rabbit-liver suspension, and extracting and purifying the compound according to the method of Kelsey *et al.* (1944). The melting point of the final product was 250°.

Chicks and dosing. The chicks used were 7–14 days old, and their body weights ranged between 50 and 80 g. A single dose of 200 mg./kg. of quinine or QDP base was given into the crop by a catheter tube. The bases were dissolved in a minimum amount of HCl. Pairs of birds from the quinine and QDP groups were killed at suitable intervals by placing

them in an atmosphere of CO_2. The blood from each pair was withdrawn from the heart and mixed in a graduated tube, heparin and citrate being added to prevent clotting. The tubes were centrifuged at about 6600 × *g* for 15 min., and the volume of plasma and red cells recorded. The concentration of quinine or QDP was determined in samples of both plasma and red cells.

Determination of quinine. Quinine was extracted and determined by the bromothymol blue method, previously described by Marshall & Rogers (1945).

Determination of QDP. Add 1 ml. of plasma or red cells to 5 ml. of distilled water in a 100 ml. glass-stoppered, narrow-necked reagent bottle. Add 1 ml. of 5N-NaOH and digest in an oven at 100° for 30 min. Cool and add 1 ml. (or sufficient to make the digest acid) of 5N-H_2SO_4, followed by 1 ml. of ammonia (sp.gr. 0·88). Cool and add 25 ml. of chloroform containing 4% ethanol. Stopper and shake the bottle for at least 1 min., but not too violently, or a persistent emulsion will be formed. It is sufficient to keep the two phases well mixed for the period of 1 min. Stand until the two layers are clearly separated, then centrifuge the bottle gently to promote complete separation. Suck off the upper aqueous layer as cleanly as possible at the water pump. Transfer the chloroform layer to a 6 × 1 in. glass-stoppered test tube and rinse out the bottle with 5 ml. of distilled water, adding this to the tube. Mix the two layers by inverting the tube several times, centrifuge, and remove the upper layer as before. Repeat the washing of the chloroform layer with a further 5 ml. of distilled water. Shake the chloroform extract with 2·5 ml. of 0·05N-HCl for at least 0·5 min., and test the upper layer to ensure that it is still acid. Centrifuge, and transfer the acid layer by means of a teat pipette to a 10 ml. flask. Repeat the acid extraction with a further 2·5 ml. of acid, and add it to the first extract. Warm the flask on a sand bath to drive off dissolved chloroform, and continue the bromothymol blue coupling procedure as for quinine.

It should be noted that if QDP is being determined in the presence of quinine, the quinine must first be removed by shaking the NaOH digest repeatedly with ether until a little of the ether extract, when shaken with dilute H_2SO_4, shows no fluorescence in the acid layer when viewed in filtered ultraviolet light.

RESULTS

The recoveries of known amounts of QDP added to chick blood are recorded in Table 1. Considering the additional technical difficulties, these values compare favourably with the recoveries of quinine by the bromothymol blue method (Marshall & Rogers, 1945).

Table 1. *Recoveries of known amounts of quinine degradation product added to chick blood*

QDP added (μg.)	QDP recovered (μg.)	Percentage recovery
5	6·0	120
5	5·1	102
10	12	120
10	9	90
25	27	108
25	23	92
35	35	100
35	35	100
50	48	96
50	47	94

The plasma and red-cell concentrations of quinine and QDP up to 7 hr. after dosing are shown graphically in Fig. 1. The red-cell concentration of both compounds was higher than the plasma concentration. The quinine curve was characterized by a sharp peak 1 hr. after dosing, followed by a rapid decline, which confirms our previous observations on the absorption of quinine (Marshall, 1945*b*). The red-cell concentration curve for QDP did not show a high initial peak, but the concentration declined less rapidly than with quinine. If the areas enclosed by the respective curves are compared, the QDP concentration over the first 7 hr. exceeds that of quinine in the ratio 1·11 : 1.

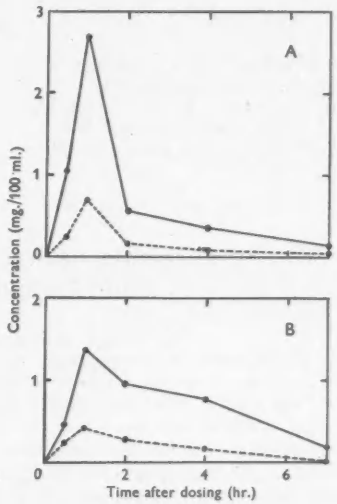

Fig. 1. Concentrations of quinine (A) and QDP (B) found in the red blood cells (continuous line) and plasma (broken line) of chicks following oral doses of 200 mg./kg. Each point represents two birds.

DISCUSSION

The results show that, over a period of 7 hr. after oral dosing, the total amounts of quinine and QDP appearing in the red cell are approximately the same. In view of the fact that the concentration curve of the degradation product falls off less rapidly than that of quinine, it may be concluded that, on considerations of red-cell concentration, QDP has the greater opportunity for being actively antimalarial than quinine. The observed lower antimalarial activity of QDP cannot, therefore, be

attributed to poor absorption in the host, but must be due to a specifically inferior activity against the malaria parasite itself.

SUMMARY

1. A method for the quantitative determination of quinine degradation product (QDP), sensitive to 5 μg., is described.

2. The red-cell concentration of QDP in chicks during 7 hr. following oral doses is approximately the same as that of an equal dose of quinine, indicating that the previously observed higher antimalarial activity of quinine is due to higher specific activity on the parasite, and not to superior absorption in the host.

The authors are indebted to Miss C. Alexander and Mr R. Neville for valuable technical assistance.

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The Relationship between Losses in Labile Liver Cytoplasm and Urinary Nitrogen Excretion

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When transferred from an adequate stock diet to a protein-free diet, female rats lose 26% and male rats 36% of their liver cytoplasm in 5 days (Kosterlitz, 1944, 1947; Campbell & Kosterlitz, 1946). It has also been observed in many species that under similar conditions the excretion of urinary N gradually falls until a relatively constant value is reached on the fifth or sixth day (Voit, 1866, 1881; cf. Kosterlitz & Campbell, 1945–6). The excretion of this extra N appears to be closely correlated with the loss of 'labile' liver cytoplasm.

METHODS

Animals. Rats of the hooded strain of the Rowett Research Institute were used. The females were 16–18 weeks and the males 13–14 weeks old. The general management was the same as that described previously (Kosterlitz, 1947).

Diets. The protein-free diets used were the basal diets of Procedures 1 and 3 described previously (Campbell & Kosterlitz, 1948*a*). Apart from vitamin supplements, 2% agar, 3% salts and 10% lard, they contained either 60% potato starch and 25% sucrose (Exps. 1, 3, 4) or 85% sucrose (Exp. 2). Whole liver concentrate was given in Exps. 1, 3 and 4 but not in Exp. 2, in which the supplements of B vitamins were wholly synthetic (Procedure 3).

Experimental procedure. Urine and faeces were collected by the method of Ackroyd & Hopkins (1916). The flasks for the collection of urine were charged with 5 ml. of 0·2 N-H_2SO_4. The funnels were washed down twice daily first with 20 and then with 25 ml. of 0·2 N-H_2SO_4, and at the end of each daily period with water. The combined urine and washings were made up to 250 ml. Urinary N was determined by the micro-Kjeldahl method (see Kosterlitz, 1947). In the experiments with sucrose, the whole faeces and, in those with potato starch, suitable samples of the finely ground faeces were ashed by the procedure of Chibnall, Rees & Williams (1943); the period of ashing, however, was reduced to 2 hr. after the clearing of the digestion mixture. A sample containing about 0·2 mg. N was used for distillation.

Calculation of the results. Each experimental period lasted for 7 days. The mean of the daily urinary N excretions on the sixth and seventh days was taken as the basal value. 'Extra N' in urine was the difference between the N actually excreted and the basal N. All values were expressed as mg./100 g. initial body weight. In the regression equations, NNS_l stands for labile non-glycogen non-lipid liver solids (mg./100 g. body weight), N_l for labile protein N (mg./100 g. body weight) and t for days of protein-free diet.

RESULTS

Faecal N *and basal urinary* N (Table 1). The values for faecal N from the second to the seventh day of the protein-free diet did not differ significantly from day to day within each experiment and were therefore pooled. In the rats which received the protein-free diet with 60% potato starch (Exps. 1, 3, 4), the mean daily faecal N values were high because of the

Table 1. *Excretion of 'extra N' in the urine, and loss of protein + nucleic acid N from the liver, during the first 5 days of a protein-free diet*

Experimental diet	No. and sex of rats	Initial body wt. (mean and range) (g.)	Results/100 g. body wt. Mean daily food intake (g. and cal.)	Mean daily faecal N (2nd–7th day) ± S.E. of the mean (mg.)	Mean daily basal urinary N (6th and 7th day) ± S.E. of the mean (mg.)	Total 'extra N' in urine (1st–5th days) (mg.)	Total loss of protein + nucleic acid N from liver (1st–5th days) (mg.)	Loss of liver N/'extra N' in urine
1. Protein-free with starch following stock diet	4 ♀	230 (216–244)	7·2 (24·5)	20·8 ± 0·7	8·4 ± 0·5	53	28	0·53
2. Protein-free with sucrose following stock diet	4 ♀	224 (197–262)	6·15 (26·6)	9·0 ± 0·6	17·1 ± 1·0	47	28	0·60
3. Protein-free with starch following stock diet	4 ♂	318 (308–338)	7·2 (24·5)	21·3 ± 0·7	9·6 ± 0·2	41	39	0·95
4. Protein-free with starch following one week 54% casein diet	4 ♂	271 (248–287)	7·1 (24·2)	24·5 ± 1·0	11·7 ± 0·6	91	59	0·65

In Exp. 4, the liver N on the 5th day of a protein-free diet following the stock diet was used for calculation instead of that on the 5th day of a protein-free diet following the 54% casein diet; therefore the calculated N loss may possibly have erred on the large side.

low digestibility of potato starch (Campbell & Kosterlitz, 1948*a*), whilst in the rats receiving the protein-free diet with sucrose (Exp. 2) the faecal N was low. On the other hand, the reverse holds for the basal urinary N values: they were low in the rats receiving potato starch and high in those receiving the sucrose diet. Thus, the presence or absence of potato starch caused only little variation in the total basal (urinary + faecal) N excretion on the last 2 days of the experimental periods; the daily means were 29·2, 26·1, 30·9 and 36·2 mg./100 g. body weight for Exps. 1–4 respectively. The slightly raised value in Exp. 4 can probably be accounted for by the fact that these rats were transferred from a high protein to the protein-free diet.

'Extra N' in urine and loss of protein + nucleic acid N from the liver (Tables 1 and 2). The daily urinary 'extra N' excretions (actually excreted N − basal N) were totalled for the first 5 days of the protein-free diet in order to obtain the total 'extra N' excreted during this period. This value showed only small variations in Exps. 1–3, in which male or female rats were transferred from the stock diet (N = 3·2%) to the protein-free diet. On the other hand, it was considerably raised in Exp. 4 in which the protein-free diet followed a 54% casein diet.

The losses of protein + nucleic acid N, which may be expected to occur from the liver during the same period, were calculated from the protein + nucleic acid N contents of livers, obtained in earlier experiments (Kosterlitz, 1947; Campbell & Kosterlitz, 1948*b*). They are summarized in Table 2. The ratios of 'loss of liver N/extra N in urine' indicate that rather more than half of the 'extra N' was derived from the protein and nucleic acid lost from the liver. If the surprisingly high value of Exp. 3 is included, the mean of the ratios is 0·68, and without it, 0·59. Non-protein N and lipid N, amounting to approximately 10% of the protein + nucleic acid N, will further increase the ratios. Thus, even if the high coefficient of variation is taken into consideration, at least 60% of the 'extra N' excreted in the urine during the first 5 days of a protein-free diet was accounted for by the N lost from the liver during this period.

Table 2. *Protein + nucleic acid N in livers of rats fed on different diets*

Diet	No. and sex of rats	Protein + nucleic acid N (mean and S.E. of the mean) (mg./100 g. initial body weight)
Stock	7 ♀	105·9 ± 2·0
4 days protein-free (starch)	4 ♀	78·0 ± 1·7
7 days protein-free (starch)	4 ♀	76·2 ± 1·7
5 days protein-free (sucrose)	5 ♀	78·0 ± 1·5
Stock	4 ♂	110·0 ± 2·1
7 days 54% casein	4 ♂	129·6 ± 5·8
5 days protein-free	4 ♂	70·5 ± 1·6

Daily variations of 'extra N' in urine and of losses of protein and nucleic acid N from the liver (Figs. 1–3). The curves obtained for the daily excretions of total urinary N in Exps. 1, 3 and 4 were not inconsistent

with the results in human beings of Martin & Robison (1922), who found that the excretions of 'extra N' in the urine diminished exponentially during the first few days of a protein-free diet. On the other hand, the curve of Exp. 2 is rather irregular.

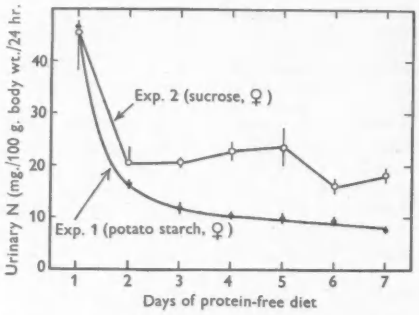

Fig. 1. Excretion of total urinary N in rats fed on a protein-free diet. (The curves were fitted by eye.) The vertical lines represent twice the standard error of the mean and in some instances, in order to avoid confusion, were drawn in one direction only.

The daily losses of protein and nucleic acid N from the liver were calculated from the regression equations for labile N (mg./100 g. body weight) on days of protein-free diet: $N_l = 31{\cdot}7\ (0{\cdot}33)^t$ for female rats, and $N_l = 42{\cdot}5\ (0{\cdot}60)^t$ for male rats. These equations were obtained from the regression equations for labile non-glycogen, non-lipid liver solids (mg./100 g. body weight) on days of protein-free diet: $NNS_l = 254\ (0{\cdot}33)^t$ for female rats, and $NNS_l = 352\ (0{\cdot}60)^t$ for male rats. The constants 254 and 352 were multiplied by $0{\cdot}916 \times 0{\cdot}136$ and $0{\cdot}885 \times 0{\cdot}136$ respectively; the factors 0·916 and 0·885 convert *NNS* to protein + phospholipin + nucleic acid, and the factor 0·136 converts the latter to protein + nucleic acid N (Campbell & Kosterlitz, 1948*a*, *c*).

The values obtained in Exps. 1 and 3 for 'extra N'/24 hr. in urine and for protein + nucleic acid N lost from the livers in 24 hr. are plotted in Fig. 3.

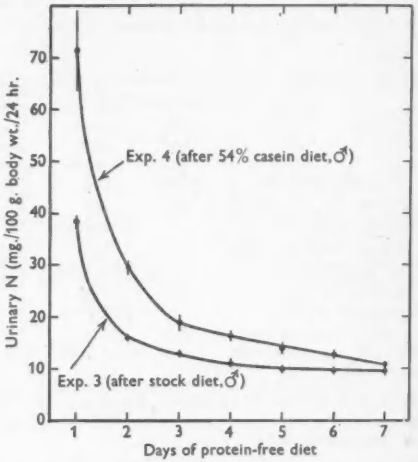

Fig. 2. Excretion of total urinary N in male rats fed on a protein-free diet. (The curves were fitted by eye.) The vertical lines represent twice the standard error of the mean.

In Exp. 1 (female rats) the two curves run close together with the exception of the value for the first day, while they show a considerable discrepancy in Exp. 3 (male rats). Whether this difference between male and female rats is significant cannot be decided with the relatively few data at present available.

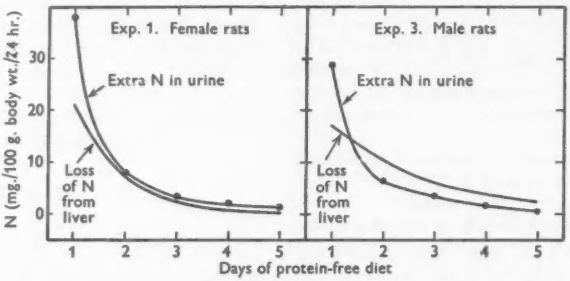
N (mg./100 g. body wt./24 hr.)
Exp. 1. Female rats
Extra N in urine
Loss of N from liver
Exp. 3. Male rats
Extra N in urine
Loss of N from liver
Days of protein-free diet

Fig. 3. Excretion of 'extra N' and loss of N from the liver. (The curves for 'extra N' were fitted by eye and those for loss of liver N were calculated from regression equations.)

DISCUSSION

The lag in the fall of urinary N to a new equilibrium when dogs were fasted, or when their protein intake was reduced, induced Voit (1866, 1881) to formulate

his theory of 'circulating protein'. This was supposed to serve as a reserve pool for protein, which was utilized by the tissues. These fundamental observations have been amply confirmed by numerous workers and extended to animals of many species, either fasted or fed on protein-free diets. Rubner (1911) thought that the 'extra N' excreted during the first few days of a protein-free diet was due to 'storage protein', which was retained or lost within 3–5 days after increasing or decreasing the protein intake. Storage protein was assumed to be unorganized, and to have as its main function the maintenance of nitrogen equilibrium with an intermittent food intake.

The experiments described in this paper, however, indicate that, while a given quantity of 'extra N' appears in the urine, at least 60%, and probably more, of this amount of N is lost from the liver. It is of interest to note that both the losses of nitrogen from the liver (Campbell & Kosterlitz, 1946, 1948*a*) and the excretions of 'extra N' (Martin & Robison, 1922) diminish exponentially during the first few days of a protein-free diet. The fact that no other organ loses nitrogen at a rate comparable with that of liver during 2 days of fasting or of a protein-free regimen (Addis, Poo & Lew, 1936*a*, *b*) supports the view that most of the 'extra N' is derived from the liver.

Conversely, Addis *et al.* (1936*b*) found that, in rats which had been transferred from a protein-free to a 74% casein diet, the first definite increase in liver protein occurred as early as 17 hr. after the protein feed. Campbell & Kosterlitz (1948*a*), who depleted rats of their labile liver cytoplasm by a protein-free regimen lasting 4 days and then fed them on an 18% casein diet, found that more than half of their labile liver cytoplasm was restored in 24 hr. and that the recovery was virtually complete in 48 hr. These findings suggest that the retention of urinary nitrogen during a period of increased protein intake can at least partly be ascribed to the formation of labile liver cytoplasm.

SUMMARY

1. An attempt has been made to correlate the excretion of 'extra N' in the urine of rats during the first few days of a protein-free regimen with the nitrogen lost from the livers during the same period.

2. At least 60% of the 'extra N' can be accounted for by the nitrogen derived from the protein, nucleic acid and other nitrogenous substances of the liver.

3. These findings make unlikely the older assumption of an unorganized 'circulating' or 'storage' protein which was supposed to be responsible for the appearance of the 'extra N'.

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An Improved Diacetyl Reaction for the Estimation of Urea in Blood

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The diacetyl or carbamido reaction of Fearon (1939) has been utilized by Ormsby (1942) and by Barker (1944) for the estimation of urea in biological fluids. Archibald (1945) has used the homologue of diacetyl, α-*iso*nitrosopropiophenone (the monoxime of benzoyl-acetyl) for a similar method which has the advantage that the colour obtained is red instead of yellow. By analogy with the Barritt (1936) reaction, in which α-naphthol is used to enhance the colour obtained from creatine and diacetyl in alkaline solution, it was considered possible similarly to enhance the diacetyl-urea reaction. The observation of Barker (1944) that thymol interfered with this reaction lent support to the hypothesis. Phenols were first tried but produced precipitates. With aromatic amines the colour produced was orange or red and not yellow, whilst diphenylamine and its derivatives produced an intense magenta colour. The reaction with *N*-phenylanthranilic acid (diphenylamine-2-carboxylic acid) has been studied in detail and adapted for the estimation of urea in blood.

EXPERIMENTAL

Effect of added substances on the carbamido reaction

The effect of the addition of various substances to the carbamido reaction was tested in the following manner. To 2 ml. of a solution of urea (0·1 mg.) 0·25 ml. of 3% diacetyl monoxime was added, followed by a few milligrams of the substance under test and finally by 4 ml. of 18 N-H_2SO_4. The solution was placed in a boiling water bath for 10 min., and immediately after removal from the bath 0·25 ml. of 1% potassium persulphate was added. The nature of the colour was then observed. With α-naphthol and thymol a precipitate was produced which dissolved on the addition of ethanol to give a red coloration. Aniline, α-napthylamine and α-naphthyldimethylamine gave a red or orange-red colour and clear solutions. With amidopyrin, an immediate violet colour was obtained which faded rapidly and was slowly succeeded by red. Diphenylamine and phenylanthranilic acid gave intense magenta colours.

The nature of this effect was further investigated by examination of the absorption spectra. Those obtained with α-naphthyldimethylamine, diphenylamine, and phenylanthranilic acid are given in Fig. 1. That produced by added α-naphthyldimethylamine is characteristic of the simple aromatic amines; the spectrum shows two maxima, one in the blue and one in the green, and the intensity of absorption is much less than that for the yellow colour obtained in the absence of added amine. With the diphenylamine derivatives there is only one absorption band in the green, and the intensity is much greater than that of the original yellow colour; in the case of phenylanthranilic acid more colour was obtained with 0·02 mg. of urea than with 0·1 mg. using the diacetyl reaction.

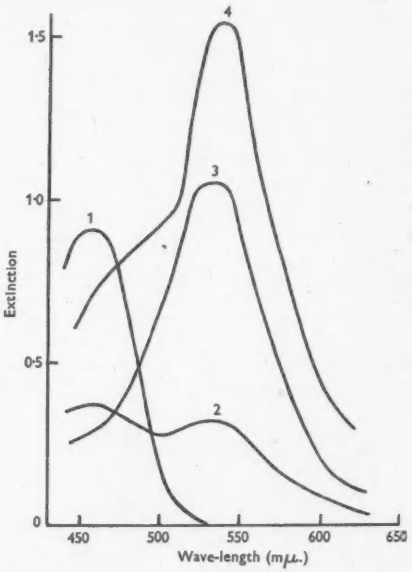

Fig. 1. Absorption spectra of solutions obtained in the diacetyl test for urea: (1) with no added amine, 0·1 mg. of urea; (2) with added α-naphthyldimethylamine, 0·1 mg. of urea; (3) with added diphenylamine, 0·1 mg. of urea; (4) with added *N*-phenylanthranilic acid, 0·02 mg. of urea.

Investigation of reaction conditions with phenylanthranilic acid

Amount of reagents. Two molecules of phenylanthranilic acid per molecule of urea were required for maximum colour production, and in order to keep the blank as low as possible minimal amounts were used. The diacetyl monoxime and potassium persulphate could be varied within wide limits without alteration of the colour produced; the amounts eventually chosen were those used by Barker (1944). The concentration of H_2SO_4 for the condensation could be varied between 9 and 14 N without alteration of the intensity of the colour, although the weaker acid solutions

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were more turbid and the colour faded more rapidly. Here again the conditions of Barker were retained by using 4 ml. of 18 N-H_2SO_4.

Time of heating. The reaction appears to reach completion after 10 min. heating in the water bath; no further increase occurs even if the heating is continued up to 30 min. (Fig. 2).

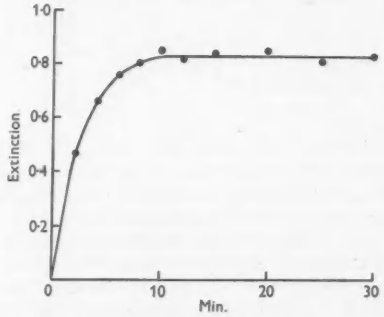

Fig. 2. Effect of varying times of heating in water bath on the intensity of colour produced in the improved diacetyl test for urea using *N*-phenylanthranilic acid.

Development of colour. After the addition of persulphate the colour develops rapidly and reaches a fairly steady value in 10–15 min. (Fig. 3); the colour then remains stable for at least 2 hr. The persulphate must be added as quickly as possible after the tubes are removed from the water bath, otherwise colour production is poor.

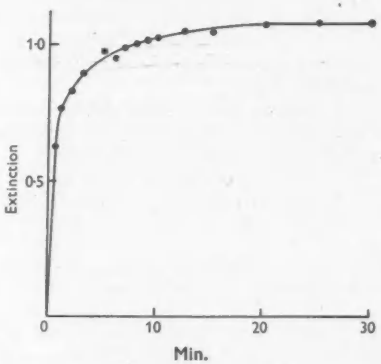

Fig. 3. Rate of development of colour after the addition of persulphate, in the reaction between urea and diacetyl in the presence of *N*-phenylanthranilic acid.

Calibration curve. Calibration curves have been prepared both with the Leifo photometer and the Spekker absorptiometer (Fig. 4). The colour obeyed Beer's law up to a urea concentration of 20 μg./ml.; beyond this the readings were erratic possibly due to difficulties in measurement of the high extinctions obtained.

Specificity. In specificity this reaction is similar to the carbamido test but with certain important differences; ammonia, glycine, histidine, tyrosine, cystine, caffeine, uric acid, barbiturates, acetamide, asparagine, creatine, sulphonamides and thiouracil all give negative reactions. Proteins and monosubstituted ureas, e.g. phenylurea, give red colorations. Semicarbazide gives a magenta colour

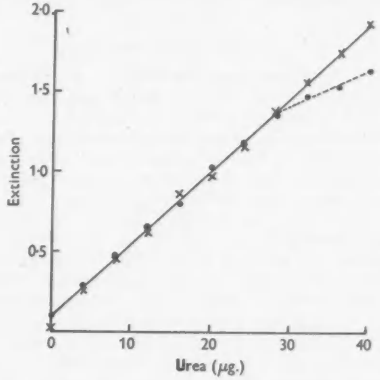

Fig. 4. Calibration curve for the improved diacetyl test with known amounts of urea. Dots are values obtained with Spekker photoelectric absorptiometer (spectrum green filter together with calorex heat filter); crosses are results with Leifo visual photometer (filter 530).

similar to that obtained with urea, different from the bright red obtained in the carbamido reaction and of lower intensity. Biuret gives a brown colour. Creatinine gives a positive reaction, and this is an important difference between the two tests; but fortunately the sensitivity in this case is only one hundredth of that with urea, so that creatinine will not interfere with the estimation of urea in biological fluids.

BLOOD UREA METHODS

Reagents

Zinc sulphate solution, 2% (w/v).

Sodium hydroxide, 0·1 N.

Isotonic sodium sulphate. 30 g./l. of anhydrous salt.

Diacetyl monoxime, 3% (w/v) in water; this solution is stable indefinitely if kept in the refrigerator and for a considerable time at room temperature.

N-Phenylanthranilic acid, 0·005 M. Phenylanthranilic acid (0·106 g.) dissolved in water containing Na_2CO_3 (0·05 g.); when dissolution is complete the solution is diluted to 100 ml.

Sulphuric acid: 18 N, made by mixing equal vols. of concentrated acid and water.

Potassium persulphate, 1% (w/v). This is stable for 2 months in the refrigerator.

Urea standards. Urea of B.P.C. purity, m.p. 130–132°, is adequate for the preparation of standards. The stock standard, 0·1% (w/v), will keep for a month or more in the refrigerator. From this the working standards are prepared at frequent intervals by suitable dilution. For visual

colorimetry three standards should be used containing respectively 3, 6 and 12 μg. urea/ml.; with the procedure for 0·1 ml. samples these are equivalent to blood ureas of 30, 60 and 120 mg./100 ml. If a photoelectric colorimeter is used a single standard containing 5 μg. urea/ml. is most suitable; this gives an extinction of *c.* 0·40 with the Ilford spectrum green filter.

Procedure for 0·1 ml. samples

Into a centrifuge tube containing 7·9 ml. of isotonic Na_2SO_4 pipette 0·1 ml. of blood. Add 1 ml. of 2% $ZnSO_4$ followed by 1 ml. of 0·1 N-NaOH, mix and filter. Sufficient filtrate is obtained to enable the determination to be performed in triplicate if desired. Pipette 2 ml. of the filtrate into a test tube. Into similar tubes pipette 2 ml. of the standards, and, if a photoelectric colorimeter is used, prepare a blank using 2 ml. of water. To each tube add 0·25 ml. of 3% diacetyl monoxime and 0·25 ml. of 0·005 M-phenylanthranilic acid followed by 4 ml. of 18 N-H_2SO_4. Mix thoroughly and place in a boiling water bath for 10 min. Remove from the bath and without cooling add at once 0·25 ml. of 1% potassium persulphate. Mix thoroughly and allow the tubes to stand for 15 min. for the colour to develop; when a visual colorimeter is used 10 min. suffices. Compare using the Ilford spectrum green filter. For a blood urea over 150 mg./100 ml. it is best to repeat the determination using 1 ml. of the filtrate, although a fairly reliable result can be obtained by diluting with water before comparing.

Procedure for 0·02 ml. samples

Into a centrifuge tube containing 2·5 ml. of isotonic Na_2SO_4 pipette 0·02 ml. of blood, add 0·2 ml. of 2% $ZnSO_4$ followed by 0·2 ml. of 0·1 N-NaOH. Mix and centrifuge. Pipette a 2 ml. sample (equivalent to 0·0137 ml. of blood) into a clean tube, taking precautions to avoid contamination by any of the precipitate, and treat exactly as described for 0·1 ml. samples. The same standards can be used, and the result is multiplied by 1·46 to allow for the extra dilution.

RESULTS

The method has been compared with the manometric hypobromite method of Van Slyke & Kugel (1933), and fairly good agreement has been obtained (Table 1). In 80% of cases the difference between the two methods was less than 3 mg./100 ml. urea, but in 10% of cases errors of 8–10 mg./100 ml. were obtained. In spite of this, these results compare favourably with other colorimetric urea methods in current use, and the method can be recommended for routine analysis where only small quantities of blood are available. It is not sufficiently accurate for urea clearance tests. It has the disadvantage that the final solution is strongly acid. Attempts to overcome this were unsuccessful since dilution with water causes precipitation of the colour complex, whilst dilution with ethanol causes the colour to fade. If acetone is used as diluent the colour develops in the absence of persulphate, but unfortunately only reaches its maximum value after 3 hr. standing.

Table 1. *Comparison of the improved diacetyl reaction for urea with the Van Slyke manometric hypobromite method*

Range of differences (mg./100 ml.)	Percentage of cases
−10 to − 6	8·6
− 6 to − 4	2·9
− 4 to − 2	17·1
− 2 to + 2	54·4
+ 2 to + 4	5·7
+ 4 to + 6	8·6
+ 6 to +10	2·9

SUMMARY

1. When the condensation of urea with diacetyl is carried out in the presence of an aromatic amine a red colour is produced, instead of the usual yellow. With diphenylamine derivatives an intense magenta colour is produced.

2. The reaction with *N*-phenylanthranilic acid has been studied in detail and a method for the determination of urea in blood has been derived.

3. The estimation can be performed with as little as 0·02 ml. of blood, and results sufficiently accurate for most clinical purposes are obtained.

I wish to thank Prof. N. F. Maclagan for advice and encouragement in the writing of this paper.

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The Chemical Conversion of Nicotinic Acid and Nicotinamide to Derivatives of *N*-Methyl-2-pyridone by Methylation and Oxidation

By W. I. M. HOLMAN and C. WIEGAND, *Medical Research Council, Department of Experimental Medicine, University of Cambridge, and Farbenfabriken Bayer, Wuppertal-Elberfeld*

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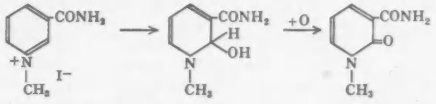
The recent work of Knox (1946) and of Knox & Grossman (1946, 1947) has shown that *N*-methylnicotinamide chloride can be oxidized by an enzyme present in rabbit liver to the amide of *N*-methyl-2-pyridone-5-carboxylic acid. These workers have also reported the isolation of the amide of *N*-methyl-2-pyridone-5-carboxylic acid from human urine after the ingestion of 0·6–0·9 g. of nicotinamide per day. It is already well known that nicotinamide is methylated in the human body and that *N*-methylnicotinamide is normally excreted in human urine. The results of Knox & Grossman (1946, 1947) therefore indicate that part of the *N*-methylnicotinamide formed from nicotinamide in the human body may be oxidized by liver enzymes to *N*-methyl-2-pyridone-5-carboxylic acid amide.

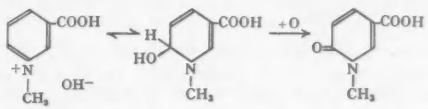
The amide of *N*-methyl-2-pyridone-5-carboxylic acid has not previously been described, but the acid itself was prepared and described by von Pechmann & Welsh (1884), who synthesized it by two methods, viz. by the action of methylamine on the methyl ester of coumalic acid, with subsequent saponification, and by evaporating 2-pyridone-5-carboxylic acid to dryness with potassium hydroxide (2 mol.) and methylating the salt with methyl iodide in methanol. Meyer (1905) prepared the acid by methylating 2-pyridone-5-carboxylic acid with methyl iodide and aqueous alkali.

Neither the method of preparation from coumalic acid and methylamine, which involves the building up of the entire ring structure, nor the method of preparation by methylation of 2-pyridone-5-carboxylic acid, explains how the *N*-methylnicotinamide is converted into the pyridone derivatives *in vitro* or *in vivo*. It seemed, therefore, of interest to see if *N*-methyl-2-pyridone-5-carboxylic acid amide can be formed from *N*-methylnicotinamide by the application of the method of Decker (1892, 1893) for the conversion of pyridinium compounds to 2-pyridones by means of alkaline ferricyanide. *N*-Methylnicotinamide was prepared by methylation of nicotinamide, and was oxidized with alkaline ferricyanide. By extraction of the reaction mixture with chloroform a compound of m.p. 216–216·5°* was isolated, which had the same elementary composition as the amide of *N*-methyl-2-pyridone-5-carboxylic acid, but which showed ultraviolet absorption properties entirely different from those reported by Knox & Grossman (1947), and which fluoresced strongly in ultraviolet light. In order to check the identity of this compound it was saponified. An acid was obtained with m.p. 183–183·5°. This acid was *N*-methyl-2-pyridone-3-carboxylic acid, for which Späth & Koller (1923) reported a melting point of 184°, and not *N*-methyl-2-pyridone-5-carboxylic acid, which has a widely different melting point (237–238° according to von Pechmann & Welsh (1884); 238–239° according to Meyer (1905)). The following series of reactions therefore occurred:



The product of the oxidation of *N*-methylnicotinamide was the amide of *N*-methyl-2-pyridone-3-carboxylic acid and not that of *N*-methyl-2-pyridone-5-carboxylic acid.

In a search of the literature a patent (D.R.P. no. 522 060) of Räth (1932) was found in which it was stated that *N*-methyl-2-pyridone-5-carboxylic acid may be prepared from nicotinic acid by methylation with methyl sulphate followed by oxidation. Nicotinic acid was therefore methylated by a method similar to that used by Winterstein & Weinhagen (1917), and the product was oxidized with alkaline ferricyanide. A good yield of *N*-methyl-2-pyridone-5-carboxylic acid (m.p. 238–238·5°) was obtained from the reaction mixture. The following reactions occurred:

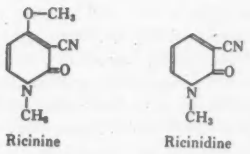


* Unless otherwise stated, the melting points given in this paper are uncorrected.

It seems that, *in vitro*, the methylation and oxidation of nicotinic acid yields *N*-methyl-2-pyridone-5-carboxylic acid, while the methylation and oxidation of nicotinamide gives the amide of *N*-methyl-2-pyridone-3-carboxylic acid. There is no evidence to suggest that mixtures of both isomers are formed in either of these reactions, since, if both amides were formed by the oxidation of *N*-methylnicotinamide, they would be extracted by chloroform* and would give, after saponification, a mixture of acids with an ill defined melting point; if both acids were formed by the methylation and oxidation of nicotinic acid, they would be precipitated by hydrochloric acid and would also give a mixture with an incorrect melting point. It was thought possible that the course of the reactions might be influenced by the nature of the methylating agent used. To test this point nicotinic acid was methylated with methyl iodide and the product oxidized, and nicotinamide was methylated with methyl sulphate and the *N*-methyl compound was oxidized. The same results were obtained as in the previous experiments, viz. nicotinic acid gave *N*-methyl-2-pyridone-5-carboxylic acid, and nicotinamide gave *N*-methyl-2-pyridone-3-carboxylic acid amide. The methylating agent, therefore, had no influence on the course of either of the reactions.

amide by a different series of reactions in the animal body from that *in vitro*, or that *N*-methyl-2-pyridone-5-carboxylic acid is first formed from nicotinic acid and is subsequently converted to the amide.

The amide of *N*-methyl-2-pyridone-3-carboxylic acid, which is formed by the oxidation of *N*-methylnicotinamide *in vitro*, but not in the animal body, has a similar structure to two compounds of interest in plant biochemistry, viz. ricinine and ricinidine. It is conceivable that plants may be capable of producing these substances from *N*-methylpyridinium compounds by oxidation.



Ricinine Ricinidine

The amide of *N*-methyl-2-pyridone-5-carboxylic acid was prepared by converting the acid to the acid chloride with thionyl chloride and by decomposing the acid chloride with aqueous ammonia; it was also prepared from the ethyl ester of the acid. The ultraviolet absorption of the amide prepared by these methods (see Fig. 1) was identical with that reported

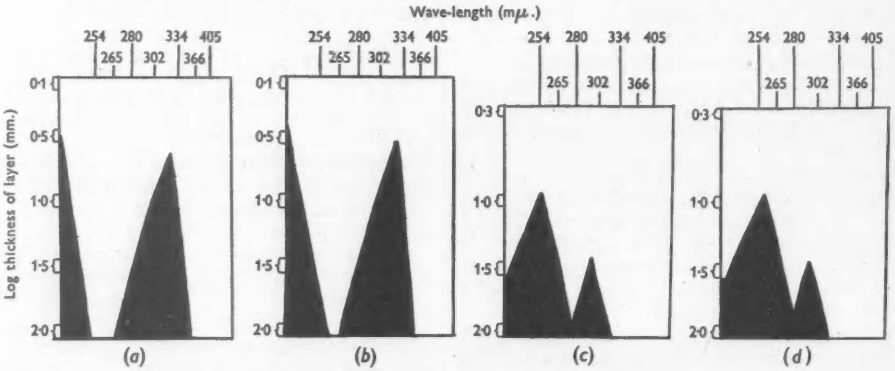


Fig. 1. Approximate diagrammatic reproductions of ultraviolet absorption photographs. (*a*) *N*-Methyl-2-pyridone-3-carboxylic acid, 0·005 % in water. (*b*) *N*-Methyl-2-pyridone-3-carboxylic acid amide, 0·005 % in water. (*c*) *N*-Methyl-2-pyridone-5-carboxylic acid, 0·001 % in water. (*d*) *N*-Methyl-2-pyridone-5-carboxylic acid amide, 0·001 % in water.

The results of the above experiments indicate either that the amide of *N*-methyl-2-pyridone-5-carboxylic acid is produced from *N*-methylnicotinamide by a different series of reactions in the animal body from that *in vitro*, or that *N*-methyl-2-pyridone-5-carboxylic acid is first formed from nicotinic acid and is subsequently converted to the amide.

The amide of *N*-methyl-2-pyridone-3-carboxylic acid, which is formed by the oxidation of *N*-methylnicotinamide *in vitro*, but not in the animal body, has a similar structure to two compounds of interest in plant biochemistry, viz. ricinine and ricinidine. It is conceivable that plants may be capable of producing these substances from *N*-methylpyridinium compounds by oxidation.

The amide of *N*-methyl-2-pyridone-5-carboxylic acid was prepared by converting the acid to the acid chloride with thionyl chloride and by decomposing the acid chloride with aqueous ammonia; it was also prepared from the ethyl ester of the acid. The ultraviolet absorption of the amide prepared by these methods (see Fig. 1) was identical with that reported by Knox & Grossman (1947). Its elementary composition agreed closely with the theoretical values. The melting points of four samples prepared by different methods (201–204°; 204–206·5°; 203–205°; 202–203° (205·5–206·5° corrected)) were definitely lower than that of 212–214°, which was found by Knox & Grossman (1947) for *N*-methyl-2-pyridone-5-carboxylic acid amide prepared enzymically from *N*-methylnicotinamide chloride. Further

* See later sections for the preparation of the amide of *N*-methyl-2-pyridone-5-carboxylic acid. Both the 2:3- and the 2:5-amides are slightly soluble in chloroform and are reasonably stable in the alkaline ferricyanide solution. Neither of the isomeric acids could be isolated from the mother liquor after extraction with chloroform.

efforts to test the purity of our specimens of the amide showed that the acid (m.p. 236–237°) could be recovered by saponification with 2·5N-sodium hydroxide, and that no change in the melting point resulted after repeated recrystallizations from various solvents, after extraction with chloroform or ether, or after previous heating of the substance to its melting point.

It is interesting to observe (Fig. 1) that there is a wide difference in ultraviolet absorption between the amide of *N*-methyl-2-pyridone-3-carboxylic acid and that of *N*-methyl-2-pyridone-5-carboxylic acid. The former compound shows strong blue fluorescence in ultraviolet light, the latter has no fluorescence. Knox & Grossman (1947) stated that *N*-methyl-2-pyridone-5-carboxylic acid amide fluoresces slightly under the conditions used in the estimation of *N*-methylnicotinamide in urine, but their preparations, which were not white in colour, may have contained traces of impurities.

The present results suggest that, in the estimation of aneurin in urine by the thiochrome method, the alkaline ferricyanide used to oxidize aneurin to thiochrome may also convert a part, at least, of the *N*-methylnicotinamide present to the fluorescent *N*-methyl-2-pyridone-3-carboxylic acid amide, which would then be extracted by *iso*butanol together with the thiochrome. The error, which Najjar & Ketron (1944) found to be introduced into the estimation of aneurin in urine by the presence of *N*-methylnicotinamide, may be attributable to a difference in the intensity of fluorescence of *N*-methyl-2-pyridone-3-carboxylic acid amide compared with that of the product of the reaction of alkali alone on *N*-methylnicotinamide, and/or to partial destruction of the latter substance by alkaline ferricyanide and incomplete formation of the former. Najjar & Ketron (1944) suggested that *N*-methylnicotinamide was converted to a pyridone by alkali and ferricyanide, but they did not isolate and identify the compound.

EXPERIMENTAL

N-*Methylnicotinamide iodide*

Nicotinamide (25 g.) was dissolved in methanol (50 ml.) and the solution boiled gently under reflux for 8 hr. with 43·7 g. methyl iodide (1·5 mol.). After removal of methanol and of the excess of methyl iodide by filtration, *N*-methylnicotinamide iodide remained as a pale yellow crystalline mass (54·3 g.) of m.p. 203–204° (cf. m.p. 204° reported by Karrer, Schwarzenbach, Benz & Solmssen, 1936).

N-*Methyl-2-pyridone-3-carboxylic acid amide*

N-Methylnicotinamide iodide (20 g.) was dissolved in water (120 ml.) in a 1 l. flask. The flask was cooled in ice. To the contents were added slowly, with constant stirring, a solution of 53 g. $K_3Fe(CN)_6$ in 170 ml. of water, and, simultaneously, a solution of 20 g. KOH in 40 ml. water. The mixture was stirred for 1 hr. The precipitated $K_4Fe(CN)_6$ was filtered off and *N*-methyl-2-pyridone-3-carboxylic acid amide was isolated from the filtrate by extracting repeatedly with chloroform. After evaporation of the combined chloroform extracts to dryness, the pale brown residue (2 g.) was purified by crystallization from methanol. The compound crystallized in the form of white needles with a faint greenish fluorescence, m.p. 216–216·5°. Späth & Koller (1923) reported a m.p. of 216° for this compound. (Found: C, 55·6; H, 5·64; N, 18·4. Calc. for $C_7H_8O_2N_2$: C, 55·2; H, 5·26; N, 18·4%.) Both the 2:3- and the 2:5-amides tend to leave a residue which contains N and C. Care must therefore be taken in the determination of N and C to ensure complete combustion.

N-*Methyl-2-pyridone-3-carboxylic acid*

N-Methyl-2-pyridone-3-carboxylic acid amide (0·5 g.) was saponified by boiling gently under reflux for 3 hr. with 2·5 ml. of 2·5N-NaOH. The solution was acidified with 2N-HCl and the precipitated *N*-methyl-2-pyridone-3-carboxylic acid was filtered off. After crystallization from water the acid was obtained as thin, pure-white needles of m.p. 183–183·5°. Späth & Koller (1923), who prepared this compound by another method, found m.p. 184°.

N-*Methyl-2-pyridone-5-carboxylic acid*

Nicotinic acid (82 g.) was covered with methanol (200 ml.), and the mixture boiled gently under reflux with 90 g. methyl sulphate. After about 0·25 hr. the nicotinic acid dissolved. After heating for 2 hr. the methanol and the excess of methyl sulphate were removed *in vacuo*. The viscous brown liquid which remained was transferred to a 3 l. flask with 200 ml. water. The flask was cooled in ice, and to the contents were added, drop by drop, with constant stirring, a solution of 440 g. $K_3Fe(CN)_6$ in 750 ml. water, and, simultaneously, a solution of 194 g. KOH in 500 ml. water. The resulting mixture was stirred for 1 hr. and was filtered to remove precipitated $K_4Fe(CN)_6$. The filtrate was acidified with 6N-HCl. The precipitated *N*-methyl-2-pyridone-5-carboxylic acid was filtered off and washed on the filter four times with cold water (yield 60 g.). It was dissolved in hot water and the solution boiled with charcoal. After crystallization from water, white needles (30 g.) were obtained with m.p. 238–238·5°. This melting point agrees well with the values of 237–238° reported by von Pechmann & Welsh (1884), and of 238–239° found by Meyer (1905).

N-*Methyl-2-pyridone-5-carboxylic acid ethyl ester*

N-Methyl-2-pyridone-5-carboxylic acid (10 g.) was esterified by heating for 6 hr. with absolute ethanol (100 ml.), through which a stream of dry HCl was bubbled continuously. The ethanol and HCl were removed by evaporation *in vacuo*. The dry residue was ground in a mortar with solid Na_2CO_3 and the ester (m.p. 72·5–74°) was extracted with benzene. It was purified by crystallization from a mixture of benzene (1 vol.) and ligroin (3 vol.); m.p. 72–73·5° (cf. m.p. 74° reported by Räth & Schiffmann, 1931).

N-*methyl-2-pyridone-5-carboxylic acid amide*

(*a*) *N*-Methyl-2-pyridone-5-carboxylic acid (10 g.) was boiled gently under reflux for 1–2 hr. with 50 ml. thionyl chloride. The excess of thionyl chloride was evaporated

in vacuo. The greenish yellow crystalline residue was ground in a mortar, and was slowly added to 200 ml. conc. NH_3, with cooling. The mixture was stirred continuously for 3 hr. *N*-Methyl-2-pyridone-5-carboxylic acid amide was precipitated as a white crystalline mass, which was filtered off (yield 5 g., m.p. 201–203·5°), and purified by crystallization from absolute ethanol (pure-white needles of m.p. 201–204°). (Found: C, 54·8; H, 5·32; N, 18·2; amide N, 9·0. Calc. for $C_7H_8O_2N_2$: C, 55·2; H, 5·26; N, 18·4; amide N, 9·2%.)

(*b*) The same procedure was used as in (*a*), except that 22·5 g. of *N*-methyl-2-pyridone-5-carboxylic acid and correspondingly greater amounts of the other reagents were taken, and the time of heating with thionyl chloride was somewhat shorter (1 hr.). The yield of the crude product was 15 g. After two recrystallizations from absolute ethanol 8·5 g. of white needles of m.p. 204–206·5° were obtained.

(*c*) The procedure was the same as in (*a*), except that the time of heating with thionyl chloride was reduced to 20 min. After crystallizing once from absolute ethanol, white needles of m.p. 202–203·5° were obtained. The product, however, contained a trace of an impurity, presumably unchanged acid, which melted at 233·5°. After treatment with a solution of $NaHCO_3$ and crystallization from absolute ethanol, no trace of the high-melting impurity remained and the melting point was sharp (202–203°, uncorrected; 205·5–206·5°, corrected). After saponification for 7 hr. with 2·5 N-NaOH, *N*-methyl-2-pyridone-5-carboxylic acid was recovered (m.p. 236–237°).

(*d*) *N*-Methyl-2-pyridone-5-carboxylic acid ethyl ester (5 g.) was heated for 8 hr. at 200° under a pressure of 63 kg./sq.cm. with 20 ml. methanol saturated with NH_3. After removal of methanol and NH_3 by evaporation on a steam bath, the residue was extracted once with benzene to remove unchanged ester. The amide of *N*-methyl-2-pyridone-5-carboxylic acid was isolated from the benzene-insoluble residue by crystallization from absolute ethanol. It was purified by a second crystallization from absolute ethanol; almost white crystals of m.p. 203–205° were obtained.

SUMMARY

1. Methods are described for the preparation of the amide of *N*-methyl-2-pyridone-3-carboxylic acid by the methylation and oxidation of nicotinamide, and for the preparation of *N*-methyl-2-pyridone-5-carboxylic acid by the methylation and oxidation of nicotinic acid. Methods are also given for the conversion of the former compound to the corresponding acid, and of the latter compound to the corresponding amide.

2. The properties of the two isomeric acids and amides are compared. The course of the reactions which occur in the formation of these compounds *in vitro* is discussed in relation to the biological formation of the amide of *N*-methyl-2-pyridone-5-carboxylic acid from nicotinamide in the human body.

We are indebted to Dr E. Merkel for the ultraviolet absorption photographs and to Dr O. Wollenberg for the elementary analyses.

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Absorption of 3-Methylglucose from the Small Intestine of the Rat and the Cat

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On the basis of the phosphorylation theory (Verzár & McDougall, 1936) certain sugars are absorbed from the intestine by an active process, one stage of which involves the phosphorylation of the sugars. Sugars so absorbed are thought to leave the gut more rapidly than those depending only on simple diffusion, and it is customary to judge whether or not a sugar is actively absorbed by comparing its rate of absorption with that of glucose. On this criterion, galactose and glucose are classed as being actively absorbed whilst the pentoses, such as xylose, are thought to enter by simple diffusion only. In the course of work on the synthetic 3-methylglucose, it became of interest to determine whether it was actively absorbed from the intestine. Two approaches were made: first, the rate of absorption from the

rat's small intestine was compared with that of glucose; secondly, the power of the gut to absorb the sugar against a concentration gradient was investigated in the cat. Both lines of inquiry indicated the existence of an active mechanism for the absorption of 3-methylglucose.

METHODS

Experiments on rats

In individual rats, under nembutal anaesthesia, two adjacent loops of the small intestine were isolated with ligatures; isotonic solutions of glucose and the sugar to be examined were placed in the separate loops and, after a given time, the contents were washed out and their reducing values determined by the Hagedorn & Jensen (1923) technique. From rat to rat the relative positions of the two solutions, proximal or distal to the stomach, were alternated in order to discount variations in the absorptive rate due to their position in the gut. The technique closely followed that described by Davidson & Garry (1941).

Experiments on cats

The general principle of these experiments consisted in maintaining a concentration of 3-methylglucose in the blood higher than that in the gut. If the sugar enters the blood only by simple diffusion, the absorption must take place as a result of the previous absorption of water to such an extent that the concentration of the sugar in the gut is greater than that in the blood. After a given time, then, any loss of sugar from the gut would have to be accompanied by a concentration in the gut equal to, or greater than, that in the blood, if simple diffusion were the only factor. The absorption of water could be by a specific process, or it could simply result from the colloid osmotic pressure of the plasma.

Thus an estimation of the amount of 3-methylglucose absorbed from an isolated loop, combined with a determination of the concentration of 3-methylglucose in the blood during the process of absorption, and in the gut fluid at the end of the absorptive period, should provide unequivocal evidence as to whether the sugar is actively absorbed, since a loss of sugar from the intestine, associated with a lower intestinal concentration than that in the blood, could not arise by a simple diffusion process. To reduce colloid osmotic influences to a minimum, the sugar was dissolved in the cat's own plasma. To ensure that the concentration in the blood would be the higher, the animal was given an intravenous injection of the sugar after the renal arteries had been tied. A large sample of blood was withdrawn, the plasma separated and 5 ml. inserted into an isolated loop; meanwhile, successive intravenous injections of the sugar were administered during the course of the experiment in amounts designed to maintain the blood level definitely higher than that in the gut. For control purposes a second loop was isolated and the animal's plasma, withdrawn before the injection of 3-methylglucose, inserted, and the absorption of glucose estimated.

The cats were cleared of intestinal worms with a dose of 10 mg. of arecoline hydrobromide in milk. The renal arteries were tied under nembutal anaesthesia. The femoral vein of one leg and the artery of the other were cannulated, and 10 ml. of blood withdrawn to provide the plasma for the control glucose absorption. A volume of 18 ml. of the 6% (w/v) 3-methylglucose in 6% gum acacia was injected and, after time had been allowed for equilibration, 10 ml. of blood were withdrawn and the plasma separated. The abdomen was then opened and the warm plasmas were inserted into adjoining isolated loops of the small intestine, the technique of Davidson & Garry (1940) being followed. During the whole of the procedure, the injection of 3-methylglucose was continued at the rate of about 2 ml. every 5 min. After a given interval the loops were removed and emptied, 0·1 ml. samples of the contents were taken for reducing-value estimations and the remainder, together with the washings of the gut, was made up to 25 ml. for Zeisel methoxyl determinations (Elek, 1939) of 3-methylglucose. Methoxyl and reducing values were also estimated on the blood samples.

In order to determine more accurately the changes in the sugar concentration of the gut fluid during the absorptive period, a second series of experiments was conducted in which a single loop was well filled with the plasma containing 3-methylglucose. The loop was emptied after a short absorption period, when at least 2 ml. of fluid remained unabsorbed. This permitted an accurate duplicate determination of the actual concentration of 3-methylglucose in the fluid. All estimations in this case were done by removing the glucose by yeast fermentation and estimating the non-fermentable 3-methylglucose by the Hagedorn & Jensen technique. The fermentation technique followed closely that described by Young (1938) and Somogyi (1927).

RESULTS

Experiments on rats

Sixteen rats were treated and the results calculated as the absorption in mg./g. of gut/hr. The results are shown in Table 1. If the rate of absorption of glucose be taken as 100 then that of 3-methylglucose is 84.

It was found that if the absorption took place in the proximal part of the intestine there was no difference between the caudal and cranial loops, whereas if more of the intestine was used and the more distal portion included there was a marked difference in the rates of absorption from the two loops, that from the caudal being appreciably less than that from the cranial loop. These differences, however, were eliminated in computing the results, since in eight rats the one sugar was in the caudal loop and in the other eight rats the position was reversed.

Table 1. *Absorption of sugars from rat intestine*

(Absorption rates as mg. sugar/g. of gut/hr.)

	3-Methylglucose	Glucose
Mean value	32·2	38·3
Standard error	2·06	1·60
Extreme ranges:		
Highest	44·7	47·0
Lowest	21·1	24·2

Difference = 15·9 %.

Experiments on cats

In the first series of experiments it was found that in each of three cats, in which the concentration of 3-methylglucose in the blood was kept above that in the gut, appreciable absorption did take place. The results are summarized in Tables 2 and 3. It will be seen from these tables that (*a*) the percentage absorption is comparable with that of glucose, and (*b*) the initial concentration of 3-methylglucose in the intestine was considerably less than that in the blood. The results giving the final concentration of 3-methylglucose were inconclusive in this series and probably not very accurate, since very little plasma remained in the intestine after the absorption period.

Table 2. *Percentage absorption of sugars in loops of cats' intestine*

Cat	3-Methylglucose	Glucose
I	30	40
II	38	35
V	50	60

Table 3. *Concentration of 3-methylglucose during absorption in the cat*

(Concentration in mg./100 ml. plasma.)

	Cat		
	I	II	V
Av. concentration of 3-methylglucose in blood during absorption	690	400	230
Initial concentration of 3-methylglucose in intestine	470	250	190

Table 4. *Concentration of 3-methylglucose during absorption in the cat*

Concentration of 3-methylglucose (mg./100 ml. plasma)		Cat VI	VII	VIII
In intestine:	Initially	219	249	231
	After absorption	189	206	178
In blood:	Before absorption	261	240	229
	After absorption	342	263	238

In the second series of experiments (Table 4) it was found that, in each of three cats in which the concentration of 3-methylglucose in the blood was maintained at or above the concentration in the intestine, an appreciable lowering of the concentration of 3-methylglucose took place.

DISCUSSION

Amongst others Cori (1925*a*, *b*) and Wilbrandt & Laszt (1933) have shown that different sugars are absorbed at different rates from the gut. In general, the hexoses are absorbed more rapidly than the pentoses. It has been suggested that the difference in the rates between these two groups of sugars is due to the fact that the hexoses (glucose and galactose) are actively absorbed, whereas the pentoses are merely absorbed by simple diffusion. Verzár & McDougall (1936) suggested that the mechanism of active absorption may be phosphorylation, a theory which is supported by the fact that glucose and galactose are phosphorylated *in vivo*, whereas the pentoses are not.

Table 5. *Absorption rates of various sugars from rat intestine*

Galactose	115	Fructose	44
Glucose	100	Pentoses	30
3-Methylglucose	84		

Table 5 shows a comparison between the rates of absorption of various sugars from the rat's intestine (Wilbrandt & Laszt, 1933) to which our results with 3-methylglucose have been added. It will be seen from this that 3-methylglucose, on the above basis, would be expected to be actively absorbed in the rat. The fact that the sugars were apparently absorbed more slowly the more distal the loop from the stomach, coupled with the evidence of Davidson & Garry (1941) that there was no difference in the rates of absorption of the pentoses in the caudal and cranial loops of the rat's intestine, suggests that the efficiency of the 'active' absorption mechanism decreases along the length of the small intestine.

In the experiments in the cat there can be little doubt that absorption did take place against a concentration gradient. It has also been shown that the concentration of sugar in the gut loop decreased during absorption so that there can be no question that absorption took place by preferential absorption of water which would upset the gradient. Bárány & Sperber (1939) have also shown that glucose can be absorbed against a concentration gradient; our results confirm this. We must, therefore, conclude that the 3-methylglucose was actively absorbed in the cat.

Previous results have shown that 3-methylglucose is not glycogenic in the rat (Campbell & Young, 1948), so that from this standpoint it would not be expected to be actively absorbed. Therefore, the

fact that it is, suggests that phosphorylation is not the mechanism of active absorption in this case. These results confirm those of Davidson & Garry (1940) in which they found that D-xylose is absorbed as rapidly as glucose from the caudal region of the small intestine of the cat. Shapiro (1947) has suggested from results with phlorrhizin and rat kidney extracts that, although phosphorylation may play a part in the absorption of sugars, it is probably far from being the only mechanism, and this is also true of the small intestines of the rat and the cat.

SUMMARY

1. In rats the rate of absorption of 3-methylglucose has been found to be about 16 % slower than that of glucose.

2. In cats 3-methylglucose has been found to be absorbed against a concentration gradient.

3. Since 3-methylglucose is not glycogenic in the rat, it is suggested that phosphorylation is not the only mechanism of active absorption either in the rat or the cat.

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Determination of Traces of Iron and Copper in Culture Media Prepared by Enzymic Digestion of Muscle Protein

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During the course of experimental work on the effect of trace metals on the toxin production of *Corynebacterium diphtheriae*, it became apparent that a very sensitive method for the determination of iron and copper was necessary. As a temporary measure, a method for iron was evolved, based on Mueller's (1938) media deferration method, consisting of adding calcium phosphate to the sample, precipitating with ammonia, and treating the precipitate with *o*-phenanthroline to develop a colour with the ferric phosphate adsorbed on the calcium phosphate. This method gave reliable and reproducible values for inorganic iron, but gives no indication of the amount of bound iron present. The problem thus resolved itself into two main steps: (*a*) incineration of the sample, and (*b*) the determination of iron and copper in the ash.

Dry ashing methods, particularly that due to Klumpp (1934), were investigated and rejected because of the significant amounts of iron and copper extracted from the ashing vessels. Silica, porcelain and pyrex crucibles all gave trouble, a fact noted by Braun & Scheffer (1940), but platinum was not tried since Bailey & McHargue (1945) stated that it gave a colour with the copper reagent similar to that given by copper itself. Wet ashing with H_2SO_4, $HClO_4$ and HNO_3 in pyrex flasks was then tried, but even after diminishing the large acid blank by redistillation of the acids, results were still erratic and high. It was not until the flasks were specially cleaned that consistent results were obtained. Wet ashing was a lengthy procedure until Smith's (1946) method was investigated, in which digestion was greatly accelerated by the use of vanadium as a catalyst. This method gave no trouble provided that the rate of heating was controlled to prevent spontaneous ignition.

Of a number of colorimetric reagents available for the determination of small quantities of iron, $\alpha\alpha'$-dipyridyl and *o*-phenanthroline appeared to be the most satisfactory from the point of view of sensitivity, stability of colour and freedom from interference, and a number of methods employing both dipyridyl (Hill, 1930; Dyer & MacFarlane, 1938; Jackson, 1938; Schulek & Floderer, 1939; Thorpe, 1941; Koenig & Johnson, 1942; Moss & Mellon, 1942; Norlin, 1943; Kitzes, Elvehjem & Schuette, 1944) and phenanthroline (Saywell & Cunningham, 1937; Fortune & Mellon, 1938; Hummel & Willard, 1938; Schaefer, 1940; Mehlig & Hulett, 1942; Borei, 1943; Bandemer & Schaible, 1944; Benne & Snyder, 1944) is described in the literature. The thiocyanate complex was rejected in view of its in-

stability and sensitivity to variation of pH, etc. (Woods & Mellon, 1941; Benne & Snyder, 1944). Phenanthroline is about one and a half times as sensitive as dipyridyl, otherwise there is little to choose between the two reagents. They both form coloured complexes with ferrous iron and give practically no colour with ferric iron, so an efficient reducing agent must be used. A number of different reducing agents has been employed, of which the most satisfactory appear to be hydroxylamine hydrochloride (Fortune & Mellon, 1938; Moss & Mellon, 1942) and *p*-hydroxyphenylglycine (Dyer & MacFarlane, 1938). Sodium hyposulphite ($Na_2S_2O_4$), though efficient, is somewhat unstable, and grades obtainable contained considerable quantities of iron.

The estimation of iron in the ash was relatively simple when phosphate was absent, but in the presence of relatively large amounts (up to 10,000 : 1) of this ion it became difficult owing to the formation of ferric pyrophosphate during ashing. Several methods have been described to overcome this difficulty, of which the methods of Schulek & Floderer (1939), as described by Moss & Mellon (1942), and Jackson (1938) appeared to be most promising. Schulek & Floderer's (1939) phosphoric acid procedure was found to give fairly good results with tryptic digests but not with papain digests; a satisfactory explanation for this was not found. Jackson's (1938) method of precipitating the iron as sulphide was not satisfactory since filters would not hold the precipitate. Adsorption of the precipitate on $Ca_3(PO_4)_2$, and PbS, although it gave better results, was not entirely satisfactory. Attempts to prevent the formation of pyrophosphate by omitting H_2SO_4 from the digestion mixture were not successful since the presence of $HClO_4$ in the residue caused the precipitation of what was presumably ferrous-phenanthroline-perchlorate, for which Smith & Richter (1944) give a solubility of 0·00103 mol./l. Destruction of the excess $HClO_4$ with hydrazine, hydroxylamine and oxalic acid was attempted but was unsuccessful owing to the large quantities of reducing agent required. Other types of reducing agents could not be used since the decomposition products had to be volatile to limit the concentration of solids in the final solution. Hydrated silica also caused trouble by adsorbing some of the coloured iron complex on standing.

The problem was finally solved by heating the ash solution to boiling with phenanthroline and hydroxylamine, when it was found that maximum colour development was obtained in less than 30 sec. As a matter of interest, this phenomenon was more fully investigated and it was found that a similar effect was obtained in the presence of other complexes; 20 μg. of Fe^{+++} were added to *c.* 0·1 g. of the complexing ion (as the Na or K salt) in 5 ml. of water, followed by phenanthroline, hydroxylamine and acetate buffer. If in 5 min. the solution had not attained the same colour as a standard, it was heated to boiling. If the colours still did not match, a tiny crystal of sodium sulphide was added and the solution reheated. The results obtained are set out in Table 1.

Table 1. *Colour development in presence of various complexing ions*

(0·1 g. Na or K salt of complex, 20 μg. Fe^{+++}, 0·25 ml. 10% (w/v) hydroxylamine hydrochloride, 0·5 ml. 0·25% (w/v) *o*-phenanthroline, 0·5 ml. 1·7 M-acetate buffer, diluted to 10 ml. with distilled water.)

Complexing ion	Maximal colour development
Benzoate	*AH*
Borogluconate	*I*
Citrate	*AH*
Ferricyanide*	*AHS*
Ferrocyanide*	*AHS*
Fluoride	*AHS*
Formate	*I*
Gluconate	*AH*
Glutamate	*I*
Glycerophosphate	*AH*
Glycollate	*AH*
Lactate	*AH*
Malate	*AH*
Maleate	*AH*
Mandelate	*AH*
Metaphosphate	*AH*
Orthophosphate	*AH*
Oxalate	*AHS*
Palmitate	Red ppt.
Phenylacetate	*AH*
Phthalate	*I*
Pyrogallate	*I*
Pyrophosphate	*AH*
Pyruvate	Incomplete
Salicylate	*AH*
Stearate	*I*
Succinate	*AH*
Sulphosalicylate	*AH*
Tannate	Pale blue ppt.
Tartrate	*AH*
Thiocyanate	*AH*
Thiodiglycollate	*AH*
Thiosulphate	*I*
Trichloroacetate	*I*

Key: *I* = immediate; *AH* = after heating; *AHS* = after heating with sulphide; * total iron liberated.

In addition to the methods noted above for the determination of copper (Braun & Scheffer, 1940; Bailey & McHargue, 1945), a trichloroacetic acid extraction method using diethyldithiocarbamate (Tompsett, 1934*a*; Cartwright, Jones & Wintrobe, 1945) and Eden & Green's (1940) method using amyl alcohol extraction, were investigated. Tompsett's (1934*a*) method proved unsatisfactory, but Eden & Green's method gave excellent results.

The determination of copper in the ash presented no difficulties since the only interfering ion likely to be encountered was iron and that was firmly bound by the phenanthroline. The use of citrate as in Eden & Green's method was continued, however, since it held calcium phosphate in solution when the solution was made alkaline with ammonia (Tompsett, 1934*b*). Originally, total iron and copper were determined on separate samples, but in the light of the methods of Parker & Griffin (1939) and Gerber, Claassen & Boruff (1942), both elements were determined in the same sample.

METHOD

Apparatus and anticontamination precautions

Great care had to be taken to exclude every chance of contamination by dust-borne and other extraneous iron at all stages of the procedure. Water, redistilled over $AgNO_3$ from a pyrex all-glass still, was used and reagents were specially purified where necessary. It was found advisable to have a dust-proof cabinet (e.g. an old balance case) where tubes, flasks, reagents, etc. could be left free from contamination at any stage of the procedure.

Pyrex narrow-necked conical flasks (250 ml.) were used for the incineration, and 10 ml. graduated centrifuge tubes for the solutions after the development of the iron colour. The copper colour was developed in 40 ml. pyrex glass-stoppered tubes. When not undergoing heating, the flasks were covered with 50 ml. pyrex beakers. The flasks were specially cleaned to prevent contamination from the glass. When new, they were treated with boiling conc. H_2SO_4 for *c.* 30 min., and then with boiling aqua regia. Before use, they were treated each time with boiling 20% (w/v) NaOH to dissolve deposited silica (which appeared to cause erratic results) and then with boiling aqua regia. After acid treatment, the flasks were rinsed successively in tap, distilled and redistilled water and then inverted on a sheet of clean glass and allowed to drain. Tubes and pipettes were cleaned by immersing in chromic-nitric acid cleaning mixture (2·5% (w/v) CrO_3 in conc. HNO_3) overnight and rinsing as above. (Chromic-nitric acid mixture was found preferable to the conventional chromic-sulphuric acid mixture. When the oxidizing properties of the acid were spent, the HNO_3 was recovered by distillation.)

For the incineration stage, a heater to accommodate six flasks was constructed from two 1 kW. electric fire elements placed end to end and mounted on a suitable fire-proof base. The elements were wired in parallel and rate of heating was controlled by a 'Variac' transformer. The elements were surrounded by a 'fence' of 'Poilite' which projected to a height of approximately 3 in. above their surface. Along one side were cut six slots into which the flask necks were placed when tilting was necessary.

To prevent contamination by dust while the flasks were on the heater, a stream of completely dust-free air was blown into them. The filter system was of the utmost importance and after experiments the following train was found to be the most efficient: main air supply, 5 lb./sq.in.; glass wool column; P. 16 'Aerox' filter; P. 27 'Aerox' filter in water; H_2SO_4 wash bottle; trap. The system was connected to a manifold with six outlets, these being connected by rubber tubing to pieces of pyrex glass tubing bent so that they could be hooked on to the neck of the flask, with the outlet about 2 in. from the bottom.

Reagents

Sulphuric acid. A.R. grade acid was distilled *in vacuo* over $AgNO_3$ to prevent volatilization of traces of $FeCl_3$.

Nitric acid. A.R. grade acid was purified as above.

Perchloric acid. The 60% (w/v) acid was purified as above and collected as the constant boiling acid of 72% (w/v) strength.

Ammonium vanadate (0·25%). This (0·25 g.) was dissolved in 75 ml. of redistilled water with heating, cooled, a few drops of conc. NH_4OH solution added and the solution diluted to 100 ml. The solution at first assumed a yellowish tint which soon faded.

Digestion mixture. The acids purified as above were mixed in the proportions by volume: H_2SO_4 (1), $HClO_4$ (1·5), HNO_3 (7·5). Vanadate solution (2·5 ml.) was added to 1 l. of this mixture, giving a concentration of *c.* 5 μg./ml. The mixture was prepared immediately before use since it tended to extract iron from glass on standing.

Phenolphthalein (0·01%). The indicator (0·01 g.) was dissolved in 50 ml. of ethanol and the solution diluted to 100 ml. with redistilled water with constant stirring. It was filtered (Whatman no. 44 paper) before use.

Ammonium hydroxide (sp.gr. 0·880). A.R. grade material was satisfactory after filtration through a Whatman no. 44 paper.

Hydrochloric acid (2N). A.R. grade acid was purified by distillation. The constant boiling mixture obtained (20·22% at 760 mm.) was diluted to 2N (360 ml. diluted to 1 l.).

Hydroxylamine hydrochloride (10%). The hydrochloride (20 g.) was dissolved in 100 ml. distilled water together with 1 g. of $Ca_3(PO_4)_2$. The solution was adjusted to pH 8 with NH_4OH solution, warmed to flocculate the precipitate, then filtered through a Whatman no. 44 paper. The solution was then adjusted back to pH 2·5 with purified HCl and diluted to 200 ml.

o-Phenanthroline (0·25%). This was made up in 0·1N-HCl.

Acetate buffer, pH 4·6. Glacial acetic acid (50 ml.) was slowly neutralized with 10N-NaOH (phenolphthalein indicator), the flask being well cooled with running water during the reaction. A further 50 ml. of glacial acetic acid were added and the solution diluted to 500 ml. The reagent was purified by the extraction method of Waring & Werkmann (1943) as follows. To the solution in a pyrex separating funnel (with an ungreased tap) was added a solution of 10–20 mg. of 8-hydroxyquinoline in 5 ml. of redistilled metal-free chloroform, and the mixture shaken vigorously for 1 min., then rotated for 30 sec. to cause the droplets to coalesce and sink. The chloroform was drawn off and the procedure repeated until this layer had only a slight yellow tint, two to three times usually being sufficient. Finally, the solution was washed with 10 ml. portions of chloroform until the chloroform extract caused no change in the colour of two to three drops of 20% ferric alum solution after vigorous shaking. The purified solution was then warmed to expel excess chloroform and finally filtered through a Whatman no. 44 paper.

Ammonium citrate (50% w/v). The reagent was purified by the extraction procedure described above for the acetate buffer.

Sodium diethyldithiocarbamate (2%). This was dissolved in redistilled water with gentle heat, and filtered through a Whatman no. 44 paper each time before use to remove deposited sulphur, etc.

iso*Amyl alcohol.* A.R. grade primary *iso*amyl alcohol could be used after one distillation, but commercial 'amyl alcohol' could only be used after purification by two to three distillations. It was readily recovered after use by drying over CaO followed by distillation.

Standard iron solution. A solution containing 100 μg. Fe^{+++}/ml. was prepared from A.R. $NH_4Fe(SO_4)_2.12H_2O$ (0·8636 g./l. 0·1N-HCl).

Standard copper solution. This solution contained 100 μg. Cu^{++}/ml. (0·3928 g. of dry, recrystallized A.R. $CuSO_4.5H_2O$/l. 0·1N-HCl).

The standards were diluted to give a combined working standard containing 20 μg. Fe^{+++} and Cu^{++}/ml. All normalities referred to are approximate.

Procedure

The samples containing 2–20 μg. of iron and copper were pipetted into clean flasks followed by 10 ml. of digestion mixture. The flasks were placed on the heater and the air tubes inserted in the necks. A gentle stream of air was passed through, and the heating carefully controlled until the initial frothing period had passed (usually 3–5 min. after boiling commenced), and the heat was then turned full on. When nitrogen peroxide fumes were evolved the flasks were removed from the heater and the air tubes removed. The heat was reduced to half, and after a few minutes the flasks were returned to the heater in the tilted position. The incineration was allowed to proceed until the solution was clear and the vigorous bubbling had ceased; then the flasks were righted, the air tubes reinserted and the heat turned to full until all the excess acid had been evaporated and a dry, white or pale yellow residue was obtained. The flasks were then removed from the heater, covered with beakers and allowed to cool.

The ash was dissolved in 3 ml. of redistilled water, two drops of phenolphthalein added and the solution neutralized with concentrated ammonia solution. Normally, not more than one to five drops were necessary. The colour was discharged and precipitated calcium phosphate redissolved by the dropwise addition of 2 N-hydrochloric acid. To prevent undue exposure of the flask contents, the iron colour developing reagents were mixed in the following proportions: hydroxylamine (1 vol.), *o*-phenanthroline (2 vol.), acetate buffer (2 vol.), and 1·25 ml. of this mixture were added to each flask. The flasks were then heated to boiling and the sides washed down by swirling the contents. Maximal colour was obtained in less than 30 sec. The solutions were then transferred to 10 ml. graduated centrifuge tubes with 5 ml. of redistilled water and the solutions diluted to 10 ml. If cloudy, the solutions were centrifuged or allowed to stand overnight in a dust-free cupboard.

The measurement of colour was carried out with a photoelectric absorptiometer against a water blank, using two Ilford filters 603 and 604 (spectrum blue-green and spectrum green) together. The peak transmission of this filter combination corresponded closely with the maximal absorption of the ferrous

Table 2. *Recoveries of iron and copper*

		Iron			Copper		
Medium no.	Fe / Cu added (μg.)	Found (μg.)	Recovered (μg.)	Recovery (%)	Found (μg.)	Recovered (μg.)	Recovery (%)
Controls	5·0	4·8	4·8	96	4·8	4·8	96
	10·0	9·6	9·6	96	9·8	9·8	98
	10·0	9·8	9·8	98	10·1	10·1	101
	10·0	9·7	9·7	97	9·8	9·8	98
BP 334*	0	2·6	—	—	22·5	—	—
	5·0	7·6	5·0	100	27·6	5·1	102
	10·0	12·2	9·6	96	32·4	9·8	98
BP 335	0	2·0	—	—	14·5	—	—
	5·0	7·1	5·1	102	19·5	5·0	100
	10·0	11·9	9·9	99	23·9	9·4	94
BP 336	0	3·1	—	—	20·5	—	—
	5·0	8·0	4·9	98	25·5	5·0	100
	10·0	12·7	9·6	96	30·2	9·7	97
BP 336*A*	0	3·6	—	—	8·2	—	—
	5·0	8·7	5·1	102	13·1	4·9	98
	10·0	13·3	9·7	97	18·4	10·2	102
BP 337	0	2·3	—	—	11·9	—	—
	5·0	7·2	4·9	98	17·1	5·2	104
BP 337*A*	0	2·3	—	—	5·8	—	—
	5·0	7·3	5·0	100	10·6	4·8	96
BP 339	0	4·0	—	—	5·4	—	—
	5·0	8·9	4·9	98	10·2	4·8	96
BP 339*A*	0	3·1	—	—	7·2	—	—
	5·0	7·9	4·8	96	12·4	5·2	104
		Mean		98	Mean		99
		Mean deviation		±1·5	Mean deviation		±2·5

* *BP* = papain digest of beef muscle.

phenanthroline complex at 508 mμ. Using 2 cm. cells, the working range was 0–20 μg. Fe, but could be extended to 40 μg. with 1 cm. cells and to 80 μg. with 0·5 cm. cells. Beer's law was obeyed and the molar extinction coefficient obtained under these conditions was 11,150.

After reading, the coloured solutions were quantitatively transferred to the glass-stoppered tubes using 5 ml. of wash water. The following reagents were then added with mixing to these solutions in the order given: ammonium citrate (1 ml.), concentrated ammonium hydroxide (1 ml.) and diethyldithiocarbamate (0·25 ml.). Exactly 10 ml. of amyl alcohol were then added and the tubes shaken for not less than 30 sec. in order to extract the copper complex completely. This colour was read after 15 min. under the same conditions as for iron, but using two Ilford 601 (spectrum violet) filters and an amyl alcohol blank. The colour obeyed Beer's law giving a molar extinction coefficient of 11,650. The ranges of working could be extended to 80 μg. as with iron.

The transmission values obtained for both colours were converted to concentration of the respective metal from a standard curve which was prepared by taking a set of standards through the whole process, excluding ashing. A blank on all the reagents was carried out with each set of determinations.

RESULTS

Table 2 gives an indication of the accuracy of the method.

SUMMARY

1. A colorimetric method is described for the determination of the iron and copper (0–20 μg.) of meat digests in the same sample. Organic matter is destroyed by rapid wet ashing with a mixture of sulphuric, perchloric and nitric acids, using vanadium as a catalyst. Special precautions against dust and reagent contamination are necessary.

2. A novel feature is the decomposition of the ferric pyrophosphate complex by heating the ash solution with phenanthroline and a reducing agent, maximum colour being developed in less than 30 sec.

3. Copper is determined as the diethyldithiocarbamate complex, after extraction with *iso*amyl alcohol.

4. Mean recoveries obtained are 98% (mean deviation ±1·5%) for iron and 99% (mean deviation ±2·5%) for copper.

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Synthesis of a Sulphur-containing Analogue of Thyroxine

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It is generally accepted that the obvious physiological effects of the thyroid hormone, as manifested by its influence on the metabolic rate, are brought about by the peripheral action of the hormone on the tissues. Although it is still not completely certain that the circulating hormone is free thyroxine itself the balance of evidence points in this direction (Harington, 1944), and this supposition is strongly supported by the recent work of Taurog & Chaikoff (1947).

In addition to its effect in stimulating the metabolism of tissues, thyroxine has an action on the anterior pituitary gland in regulating the output of thyrotrophic hormone by the latter organ. It is this effect which is responsible for the hypertrophy of the thyroid gland that results from treatment with one of the antithyroid drugs of the thiouracil type; under the action of these drugs thyroxine synthesis in the thyroid gland is blocked, with resulting diminution of the concentration of circulating thyroid hormone in the body; the response of the anterior pituitary gland to this situation is to release more thyrotrophic hormone, which in turn produces overgrowth of the thyroid gland. Conversely, if circulating thyroid hormone is increased by exogenous administration, thyrotrophic hormone output is diminished, and the thyroid gland passes into a resting state.

The maintenance of normal conditions in the body, so far as thyroid hormone is concerned, must therefore depend on the precise relationship between the two actions of this hormone, namely its effect on tissue metabolism on the one hand and its effect on the anterior pituitary gland on the other. The question arises as to whether it is possible to dissociate these two effects.

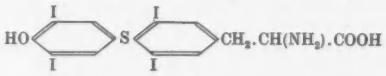
If such a dissociation could be brought about the results would be of considerable interest. A compound possessing the characteristic effect of thyroxine on tissue metabolism but, lacking its action on the anterior pituitary gland would, if administered exogenously, produce a greatly exaggerated stimulation of metabolism, since its own intrinsic action in this direction would be unaccompanied by the restraint of thyroid gland activity imposed by diminution of thyrotrophic hormone output. On the other hand, a compound acting on the anterior pituitary gland in the same way as thyroxine, but lacking the power of the latter to stimulate tissue metabolism, would bring about inhibition of thyroid activity and an overall reduction in the metabolic rate.

The latter possibility is the more interesting, for, with the aid of such a compound, thyroid activity could be controlled in a more rational manner than it can be by the so-called antithyroid drugs. The depression of thyroid function brought about by these drugs necessarily carries with it the likelihood of simultaneous production of physical hypertrophy of the gland; a compound having the properties discussed above should, however, diminish thyroid function, in the sense of thyroxine output, without entailing hypertrophy of the gland; its action should indeed cause a hyperactive gland, such as that existing in Graves's disease, to revert to the normal state. The therapeutic potentialities of such a compound are obvious.

Consideration on these lines points to the desirability of examining the physiological action of compounds closely allied in chemical composition to thyroxine itself. Certain substances of this kind are already known, namely the analogues of thyroxine in which the iodine atoms are replaced by bromine or chlorine (cf. Schuegraf, 1929). It was shown many years ago by Gaddum (1929–30) that 3:5:3′:5′-tetrabromothyronine had the same qualitative effect as thyroxine, although in much lower degree. The action of this compound, and also that of the chlorine-containing analogue, have recently been investigated in human myxoedema (Lerman & Harington, 1948); both have proved to be capable, in appropriate doses, of restoring the metabolism of a myxoedematous patient to the normal level. In so far as these substances exhibit the characteristic peripheral effect of thyroxine, therefore, they do not seem to be promising from the present point of view; however, their action on the anterior pituitary gland remains to be investigated and the possibility exists that this may predominate over the peripheral effect; if such be the case it should be possible, by their administration at the correct level of dosage, to produce a diminution rather than an increase in the rate of metabolism.

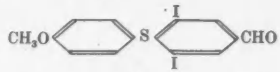
In the meantime it has seemed desirable to make available for study quite a different type of thyroxine analogue and for this purpose the *compound* (I) was selected; it will be seen that this is identical with thyroxine save that sulphur replaces oxygen as

the element linking the two benzene rings. It appeared likely on general grounds that the sulphur-containing derivative could be synthesized on the same lines as thyroxine itself (cf. Harington & Barger, 1927). So far as the early stages of the



I

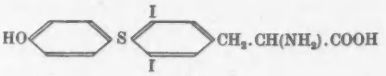
synthesis are concerned this indeed proved to be the case, and it was possible, starting with 4-methoxythiophenol and 3:4:5-triiodonitrobenzene, to obtain 3:5-*diiodo*-4(4′-*methoxy*)*phenylsulphidobenzaldehyde* (II) by a series of reactions precisely similar to those



II

employed in the thyroxine synthesis; the yields in the diphenyl sulphide series were in fact rather better than in the diphenyl ether series.

Quite unexpectedly, however, the synthesis of 3:5-*diiodo* - 4 (4′ - *hydroxy*) *phenylsulphidophenylalanine* (III) from the aldehyde (II) could not be carried out



III

by the modified Erlenmeyer method (cf. Harington & McCartney, 1927). Condensation of the aldehyde (II) with hippuric acid gave the *azlactone* in excellent yield, and this in turn could be smoothly converted into the corresponding *benzamidocinnamic acid*, but when either this compound or the azlactone itself was boiled with hydriodic acid, acetic acid and red phosphorus, elimination of the iodine atoms readily occurred and the product consisted of a mixture of partly and completely deiodinated material. Many attempts were made to circumvent this difficulty by modification of the conditions, but without success; even treatment with dilute hydriodic acid in acetic acid, as employed by Lamb & Robson (1931) in the stepwise conversion of azlactones and benzamidocinnamic acids into amino-acids, failed to give a satisfactory result.

In the case of diiodothyronine (Harington & McCartney, 1927) the analogous conversion of the azlactone or benzamidocinnamic acid can be carried out in good yield, although even here more recent experience has shown that too prolonged boiling with the hydriodic-acetic acid mixture and phosphorus causes more or less deiodination. It is clear, however, that the lability of the iodine atoms in the corresponding diphenyl sulphide derivative is much greater, and an alternative route from the aldehyde to the amino-acid had to be found.

Success was attained quite simply as follows. The aldehyde (II) was reduced to the corresponding *alcohol* by means of aluminium *iso*propoxide; the alcohol was converted into the *chloride* and the latter condensed with ethyl acetamidomalonate; appropriate treatment of the resulting *ester* with a mixture of hydrobromic and acetic acids gave the hydroxydiiodoamino-acid (III) in satisfactory yield and final iodination in ammoniacal solution afforded the desired thyroxine analogue (I).

In acute experiments the thyroxine analogue was non-toxic to mice in doses up to 0·5 g./kg. (subcutaneous) and 0·25 g./kg. (intravenous). Tested on tadpoles (*Xenopus laevis*) it accelerated metamorphosis, its activity in this respect being approximately one fifth of that of thyroxine. More detailed studies of the physiological action are now in progress.

EXPERIMENTAL

3:5-*Diiodo*-4(4′-*methoxy*)*phenylsulphidonitrobenzene*. 3:4:5-Triiodonitrobenzene (86·5 g.) was dissolved in dioxan (175 ml.) and 4-methoxythiophenol (26 ml.) (Suter & Hanson, 1932) was added. The mixture was vigorously stirred at room temperature while NaOH (17·3 ml. of 10N) was run in. Heat was developed and crystalline material soon began to separate. Stirring was continued for 30 min. after which a sample diluted with water had a neutral reaction.

Water (about 2 vol.) was then added with further stirring; the precipitate, which was first oily but soon crystallized, was collected and washed with water. It was recrystallized from acetic acid, from which it separated in long bright yellow needles, m.p. 140°. The melting point was unchanged by further crystallization from methyl ethyl ketone. Yield 78 g. (92%). (Found: S, 6·2; I, 49·7. $C_{13}H_9O_3NSI_2$ requires S, 6·2; I, 49·5%.)

3:5-*Diiodo*-4(4′-*methoxy*)*phenylsulphidoaniline*. Stannous chloride (110 g. of the dihydrate) was suspended in acetic acid (500 ml.) and brought into solution by saturation with HCl while being stirred and heated to about 70° on a water bath. The above nitro compound (73·5 g.) was then added as a suspension in acetic acid (250 ml.). The water bath was brought to the boil, vigorous stirring and rapid introduction of HCl being continued. The reaction appeared to be complete in about 45 min. as shown by saturation with HCl and no further separation of stannichloride; after a further 15 min. the mixture was cooled and the stannichloride of the base collected and washed with cold acetic acid. It was then ground up and shaken well with NaOH solution (1500 ml. of 3N) and the base collected and washed with water. The base was dissolved in ether (3000–3500 ml.) and the ethereal solution washed with 200 ml. of 2N-NaOH, dried over NaOH pellets and saturated with HCl. The precipitated hydrochloride was collected, washed with ether and dried. The yield was 63 g. (84%). After recrystallization from ethanol containing a little HCl it had m.p. 166–168°. (Found: N, 2·5; S, 5·6. $C_{13}H_{11}ONSI_2.HCl$ requires N, 2·7; S, 6·2%.)

The free *base* was prepared by dissolving the hydrochloride in absolute ethanol, adding excess of ammonia to the hot solution and diluting with water. After recrystallization from butanol it formed colourless fern-shaped crystals, m.p. 177°. (Found: N, 2·7; S, 6·9. $C_{13}H_{11}ONSI_2$ requires N, 2·9; S, 6·6%.)

3:5-*Diiodo*-4(4′-*methoxy*)*phenylsulphidobenzonitrile*. A solution of cuprous cyanide was prepared by mixing NaCN (213 g.) dissolved in water (490 ml.) with $CuSO_4.5H_2O$ (250 g.) in water (970 ml.). The hydrochloride of the above base (52 g.) was powdered and suspended in acetic acid (520 ml.); the stirred suspension was treated with amyl nitrite (15 ml.) which was added quickly; the solid passed into solution within a few minutes. The solution thus prepared was added slowly with stirring to the cooled cuprous cyanide and the mixture warmed to about 80° to complete the reaction. The precipitate was collected, washed with water and boiled out with three successive quantities of chloroform. The chloroform extracts were dried and evaporated to a low bulk, when crystallization set in. After thorough cooling the crystals were collected and recrystallized from methyl ethyl ketone, the solution being cooled in a freezing mixture. There was thus obtained 28·4 g. (55%) of a product, melting at 149° after some sintering, which was pure enough for the next reaction. After further recrystallization from methyl ethyl ketone the compound formed pale yellow hexagonal prisms, m.p. 150°. (Found: N, 2·5; S, 6·5; I, 51·4. $C_{14}H_9ONSI_2$ requires N, 2·8; S, 6·4; I, 51·5%.)

3:5-*Diiodo*-4(4′-*methoxy*)*phenylsulphidobenzaldehyde* (II). $SnCl_2$ (60 g.) was suspended in dry ether (220 ml.) and brought into solution by stirring and saturating with HCl. A solution of the above nitrile (28·15 g.) in dry chloroform (75 ml.) was added, stirring and introduction of HCl being continued for 5 hr. After standing overnight the mixture, which consisted of an upper pale yellow and a lower deep orange-coloured layer, was treated with crushed ice and evaporated *in vacuo* until all the organic solvents were removed. The solid residue was heated with dilute HCl to the boiling point and, after cooling, the crude aldehyde was taken up in chloroform; the chloroform solution was shaken with aqueous $Na_2S_2O_5$ (300 ml. of 30% (w/v)) and the bisulphite compound which separated was collected, washed with ether and decomposed by warming with dilute H_2SO_4. The aldehyde was crystallized from acetic acid from which it separated in large pale yellow needles, m.p. 134° (unchanged by further recrystallization). The yield was 18·6 g. (66%). (Found: S, 6·3; I, 50·6. $C_{14}H_{10}O_2SI_2$ requires S, 6·45; I, 51·2%.)

Azlactone. The above aldehyde (11·1 g.), hippuric acid (4 g.) and fused sodium acetate (11·1 g.) were ground up together; the mixture was treated with acetic anhydride (70 ml.) and heated on the boiling water bath for 15 min. with intermittent stirring. After cooling, the mixture was stirred with a large volume of water containing some H_2SO_4 until the acetic anhydride was decomposed. The product was collected, washed with water and dried. The yield was 13·9 g. (97%) of practically pure azlactone, m.p. 224°. After recrystallization from acetic acid, in which it is very sparingly soluble, it formed yellow needles, m.p. 228°. (Found: C, 43·0; H, 2·35; S, 5·0. $C_{22}H_{13}O_2NSI_2$ requires C, 43·2; H, 2·5; S, 5·0%.)

3:5-*Diiodo*-4(4′-*methoxy*)*phenylsulphidobenzamidocinnamic acid*. The azlactone (9·5 g.) was added to a boiling mixture of ethanol (950 ml.) and 5N-NaOH (50 ml.); the solid passed rapidly into solution and boiling was continued for 10 min. after this had happened. The solution was acidified while hot with concentrated HCl and diluted with an equal volume of water. This caused separation of the product in gelatinous form. The acid was brought into solution with ammonia and the boiling solution again acidified; boiling was continued for a few minutes after which the precipitated acid was almost completely crystalline. The yield of acid, collected after cooling, was quantitative. Recrystallized from aqueous dioxan it formed bunches of pale yellow needles, m.p. 245° (decomp.). (Found: S, 4·7. $C_{23}H_{17}O_6NSI_2$ requires S, 4·9%.)

As already indicated, all attempts to convert either this acid or the azlactone into 3:5-diiodo-4(4′-methoxy)phenylsulphidophenylalanine were frustrated by the ease with which the iodine atoms were eliminated on treatment with hydriodic acid and red phosphorus.

3:5-*Diiodo*-4(4′-*methoxy*)*phenylsulphidobenzyl alcohol*. 3:5-Diiodo-4(4′-methoxy)phenylsulphidobenzaldehyde (5 g.) was heated to gentle boiling under a reflux air condenser with a mixture of aluminium *iso*propoxide (20 ml. of approximately N solution in *iso*propanol), toluene (10 ml.) and *iso*propanol (10 ml.); a slow stream of N_2 was bubbled through the solution until evolution of acetone ceased (about 3½ hr.). The solution was evaporated to dryness *in vacuo* and the residue was ground up with dilute HCl, collected and washed with water. The moist filter cake was crystallized from acetic acid, in which it was very soluble; the product, which separated in colourless prismatic needles, m.p. 132°, amounted to 4·5 g. (90%). (Found: S, 6·5; I, 50·3. $C_{14}H_{12}OSI_2$ requires S, 6·4; I, 51·0%.)

3:5-*Diiodo*-4(4′-*methoxy*)*phenylsulphidobenzyl chloride*. The above alcohol (3 g.) was dissolved in warm dry chloroform (15 ml.). The solution was cooled in ice water and to the resulting crystalline paste PCl_5 (1·5 g.) was added. The mixture was shaken with continued cooling until practically all the solid had passed into solution; it was then kept at room temperature for 2 hr. A small amount of precipitate was removed by filtration and the filtrate was diluted with about 2 vol. of light petroleum (b.p. 60–80°); this produced a slight brown flocculum which was removed. The filtrate was then washed with water, $NaHCO_3$ solution (thoroughly) and again with water; it was dried over $CaCl_2$ and evaporated *in vacuo*. The residue crystallized almost at once; it was recrystallized from a small volume of acetic acid from which it separated in colourless rhombs, m.p. 110°. One further recrystallization raised the m.p. to 112°. The yield was 2·5 g. (83%). (Found: S, 6·3; I, 48·7. $C_{14}H_{11}OSCII_2$ requires S, 6·2; I, 49·1%.)

Ethyl 3:5-*diiodo*-4(4′-*methoxy*)*phenylsulphidobenzylacetamidomalonate*. A solution of sodium (0·1 g.) in ethanol (5 ml.) was prepared and, after cooling, ethyl acetamidomalonate (0·84 g.) was introduced; there was then added a solution of the above chloride (2 g.) in dioxan (5 ml.). The mixture was boiled under reflux; separation of NaCl soon began, and the reaction was complete in 2½ hr. On cooling and dilution with water an oily precipitate was produced which soon crystallized; this was collected, washed with water and recrystallized from ethanol. The ester formed colourless prismatic needles, m.p. 138–140°. The yield was 2·25 g. (83·5%). One further recrystallization from ethanol raised the m.p. to 144°. (Found: C, 39·8; H, 3·8; S, 4·5; I, 36·2. $C_{23}H_{25}O_6NSI_2$ requires C, 39·6; H, 3·6; S, 4·6; I, 36·4%.)

3 : 4 - *Diiodo* - 4(4′ - *hydroxy*)*phenylsulphidophenylalanine* (III). Prolonged boiling of the above ester with mixtures of hydrobromic and acetic acids caused considerable darkening with formation of tar and correspondingly poor yields of the desired amino-acid. It was assumed that of the three hydrolytic reactions occurring, deacetylation, de-esterification and demethylation, the last would be the slowest; an experiment was therefore carried out to determine the minimum time required for this reaction. In this experiment anisic acid was boiled under reflux with forty parts of a mixture of equal volumes of HBr (6 N) and acetic acid, samples being withdrawn at intervals for estimation of the phenol (*p*-hydroxybenzoic acid) content. The results showed that demethylation was substantially complete (95–100%) in 2 hr.

The ester (6 g.) was therefore boiled under reflux for 2 hr. with a mixture of HBr (120 ml. of 6 N) and acetic acid (120 ml.). The solution was evaporated to dryness *in vacuo* and the largely crystalline residue was boiled with water (about 250 ml.) and filtered hot through a wet paper to remove tar. The filtrate was brought to the boil and cautiously neutralized to about pH 5·0 with sodium acetate. The heavy granular precipitate which separated was collected after cooling, washed with water and redissolved in a mixture of 80 ml. ethanol and 20 ml. N-NaOH. On boiling this solution and adding acetic acid rapid separation of the amino-acid occurred in tufts of colourless needles, m.p. 262° (decomp.). The yield was 3·05 g. (65%). (Found: C, 33·0; H, 2·4; S, 6·1; I, 46·9. $C_{15}H_{13}O_3NSI_2$ requires C, 33·25; H, 2·4; S, 5·9; I, 47·0%.) The compound gave a ninhydrin reaction with a reddish hue, and a positive Millon reaction. It was practically insoluble in water and only very sparingly soluble in dilute aqueous mineral acids.

3 : 5 - *Diiodo* - 4(3′ : 5′ - *diiodo* - 4′ - *hydroxy*)*phenylsulphidophenylalanine* (I). The diiodoamino-acid (3·6 g.) was dissolved in ammonia (144 ml. of sp.gr. 0·880); this solution was treated drop by drop with I_2 in KI (6·2 ml. of 4·31 N). A small amount of tarry material separated towards the end of the reaction. After all the iodine had been added the solution was evaporated to dryness *in vacuo* and the residue, which was already partly crystalline, was dissolved in boiling 0·1 N-Na_2CO_3 (about 1000 ml.); a little insoluble matter was removed by filtration and the filtrate was brought to about pH 5·0 at the boiling point by addition of dilute HCl. The partly crystalline precipitate was collected after cooling and dissolved in a mixture of 160 ml. ethanol and 40 ml. N-NaOH; the solution was brought to the boil and cautiously treated with acetic acid. Just before neutrality was reached a pigmented flocculent precipitate separated; this was removed by filtration as rapidly as possible, the filtrate was brought to the boil and the acidification with acetic acid completed. The amino-acid separated in rosettes of tiny needles. The yield was 3·6 g. (68%). For complete purification the crystallization was repeated as above, again with removal of a slight pigmented flocculum, and the product was then obtained in sheaves of small but well-formed needles which were almost colourless; m.p. 226° (decomp.). (Found: C, 23·5; H, 1·6; S, 4·0; I, 64·0. $C_{15}H_{11}O_3NSI_4$ requires C, 22·7; H, 1·4; S, 4·0; I, 64·0%.) The compound resembled thyroxine closely in its general physical properties and solubilities; it gave with intensity the colour reaction with nitrous acid and ammonia which is given by thyroxine and many other *o*-diiodophenols.

SUMMARY

1. The possibility is discussed of controlling thyroid function by means of compounds inhibiting the output of thyrotrophic hormone from the anterior pituitary gland.

2. It is pointed out that such compounds may occur among chemical analogues of thyroxine. The synthesis is described of 3:5-*diiodo*-4(4′-*hydroxy*)-*phenylsulphidophenylalanine*, a sulphur-containing analogue of thyroxine, which is designed for the test of this hypothesis.

After this synthesis had been planned and begun I learnt by chance that Dr Paul Block, junr., had done some preliminary experiments on similar lines which circumstances had compelled him to interrupt. Since Dr Block did not have the particular theoretical interest in the work which is indicated by the introduction to this paper, and since the early resumption of his experiments was impossible, it was agreed that the synthesis should be completed in my laboratory. I wish to express my appreciation of Dr Block's attitude in this matter, and also of his generosity in giving me valuable information about the most favourable conditions for carrying out the first reaction.

I am indebted to Dr W. L. M. Perry for carrying out the toxicity tests and to Dr A. S. Parkes for the tests on tadpoles. My thanks are also due to Mr W. Hickford for technical assistance.

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The Action of Alkalis on Wool

3. THE ACTION OF ALKALIS ON DEAMINATED WOOL

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Following the isolation of lanthionine from alkali-treated wool by Horn, Jones & Ringel (1941), Cuthbertson & Phillips (1945) demonstrated that two major reactions of the combined cystine occurred during treatment of wool with dilute alkalis, one leading to the formation of lanthionine and the other by a reaction analogous to one previously studied by Bergmann & Stather (1926) leading to the production of combined α-aminoacrylic acid. They also found that reactions previously suggested by Speakman (1936) and Phillips (1936), giving rise to —S.NH— and —CH=N— linkages in the fibre, did not occur.

These latter linkages had the great merit, however, of explaining certain aspects of the physical behaviour of wool fibres which could not be readily explained on the basis of the two reactions formulated by Cuthbertson & Phillips (1945). Thus, if a wool fibre is extended in cold water and boiled in a mildly alkaline buffer for some time, it remains permanently elongated and will not return to its original length even when it is boiled in water in the absence of tension. If a deaminated fibre is similarly treated, however, far from acquiring a permanent set, it will 'supercontract' in boiling water to a length less than its original unstretched length. Speakman's chemical theory of set explained the setting of the untreated fibre as being due to the formation of stabilizing —S.NH— linkages, whilst the impossibility of forming such linkages in deaminated fibres leads to supercontraction. If the setting of wool fibres by treatment with dilute alkali is attributed to the formation of lanthionine cross linkages, no explanation is afforded of the failure to set and the supercontraction of deaminated fibres. Two hypotheses may, however, be considered: (*a*) the action of alkali on deaminated fibres is different in untreated and deaminated wool, or (*b*) the mechanism of set is fundamentally physical rather than chemical. Evidence is presented in the present paper to show that the combined cystine of deaminated wool does behave in a different manner towards alkalis. Under the conditions used by Speakman in his investigation of the physical properties of fibres, however, the difference in reactivity is slight, and to this extent the results support the physical mechanism of set proposed by Blackburn & Lindley (1948).

METHODS

The wool used was a commercially scoured and dry-combed merino wool of 64's quality purified by successive extraction with benzene, ethanol and water. All analyses were carried out on wool conditioned in a controlled atmosphere at 21° and 65% relative humidity, but are recorded as percentages of the dry weight. Total S was determined by a modification of the Benedict-Denis method (Barritt, 1934); $SO_4^{=}$-S and S oxidizable to $SO_4^{=}$ were determined on the acid hydrolysate of the wool (1 g. hydrolyzed for 4 hr. with 5N-HCl) by Blumenthal & Clarke's (1935) method; thiol and disulphide S by the method of Shinohara (1935*a*, *b*), using a Spekker absorptiometer; methionine S was calculated from a determination of 'volatile iodide' using Baernstein's (1936) technique. Total N was determined by micro-Kjeldahl procedure; amino N on intact wool by the method of Lindley & Phillips (1947); arginine by Vickery's (1940) method. Cysteic acid was semi-quantitatively estimated by comparing the intensity of colour and spot size given on a filter paper strip by a known amount of wool hydrolysate (250 μg.) with known amounts of cysteic acid using phenol: 0·1% NH_3: coal gas (Consden, Gordon & Martin, 1944, 1946). For lanthionine, a similar method was used after removal of aspartic and glutamic acids by Amberlite I.R. 4 at pH 3–4 (Consden, Gordon & Martin, 1948) using 5 μl. of hydrolysate equivalent to 80 μg. of wool. Some interference from cystine still occurs after removal of glutamic and aspartic acid, however, and in some of the later determinations the hydrolysate was treated with Br_2. This oxidizes cystine to cysteic acid and thus prevents interference. The sulphoxide of lanthionine also formed by the action of Br_2 has a somewhat lower R_F value than lanthionine so that the danger of overlapping with the serine spot no longer exists. (It is hoped to develop a quantitative method for lanthionine on these lines using starch columns (Elsden & Synge, 1944).) The wool was deaminated by the method of Speakman & Stott (1934).

Alkali treatment of the deaminated wools. Deaminated wool (3 g.) was boiled under reflux for varying periods in 375 ml. of the following buffers: pH 8·2 (8·06 g. boric acid plus 6·7 g. sodium borate/l., observed pH 8·22); pH 9·18 (0·05M-sodium borate, observed pH 9·18); pH 10·0 (2·58 g. boric acid and 4·4 ml. of 40% (w/v) NaOH in 1 l., observed pH 10·4; solution was brought to pH 10 by addition of HCl). In each case the wool was washed with water, dried and conditioned before analysis.

The measurement of the setting properties of deaminated wool was made in a frame similar to that used by Speakman (1936).

RESULTS

Analysis of deaminated wool

Table 1 lists analyses of untreated wool and wool which had been deaminated up to a maximum of four times under the conditions used by Speakman & Stott (1934). The results show that some oxidation of cystine S has occurred in the first deamination, but that little further attack takes place on repeated deamination. It can also be seen that the amino N of the wool is reduced substantially, but not completely, by repeated deamination. This is somewhat surprising since the method of calculation (extrapolation back of the straight-line portion of the curve relating time and nitrogen evolved (Rutherford, Harris & Smith, 1937)) suggests that liberation of the amino N (excluding arginine) is complete in 30 min. treatment with nitrous acid (cf. Fig. 1). Moreover, two-dimensional chromatography on filter paper confirmed the fact that lysine was absent from a hydrolysate of once deaminated wool, but a strong spot corresponding to α-amino-ε-hydroxycaproic acid was observed. Lindley & Phillips (1947) had no difficulty in acetylating all the amino groups of wool at room temperature. The arginine content of once deaminated wool was found to be 4·8 % compared with 10·2 % for untreated wool. It would appear, therefore, either that the method of calculating the amino N figures does not apply to deaminated wool or that the peptide bonds of deaminated wool are photosensitive. It was noted that tyrosine was absent from two-dimensional chromatograms of deaminated wool.

Table 1. *The S distribution and amino N content of wool subjected to repeated deamination treatments*

No. of deamination treatments	Total S (%)	Thiol + disulphide S (%)	Br-oxidizable S + SO_4^{--} as S (%)	Methionine S (%)	Amino N (mg./g. wool)
0	3·68	3·05	0·26	0·15	3·05
1	3·67	2·51	0·22	0·15	1·27
2	3·67	2·49	0·36	0·15	1·26
3	3·63	2·49	0·34	0·15	1·26
4	3·49	2·43	0·33	0·15	1·09

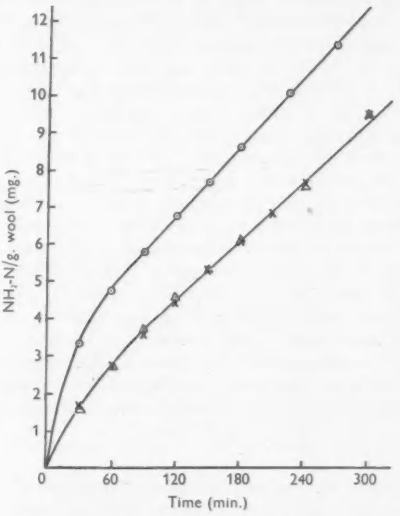

Fig. 1. The rate of liberation of nitrogen from deaminated wool on treatment with nitrous acid. ⊙—⊙, untreated wool; ×—×, deaminated wool; △—△, thrice deaminated wool.

It will be seen that once deaminated wool contains 0·79 % S unaccounted for either as cystine, methionine, sulphate S or Br-oxidizable S ('non-disulphide S'), compared with 0·22 % non-disulphide S for untreated wool. Of this S, 0·2 % was found to be present as cysteic acid formed by oxidation of cystine during deamination, and both untreated and deaminated wools contained about 0·2 % of lanthionine S, but the remaining 0·4 % of non-disulphide S of deaminated wool remains unidentified.

Physical properties of deaminated fibres

Speakman (1936) found that deaminated fibres spontaneously contracted to a length less than their original length on immersing in boiling 0·05 M-borax solution. In order to confirm that our deaminated fibres behaved similarly, two deaminated and two untreated fibres were measured before and after boiling in 0·05 M-borax solution for 10 min. The deaminated fibres showed supercontractions of 30 and 23 %, compared with 1·6 and 1·9 % for the untreated fibres, thus confirming Speakman's observations.

Speakman also noted that deaminated fibres would not take a permanent set in steam or borax; thus, two fibres, one untreated and the other deaminated, were extended 36 % and steamed in the stretched condition for 6 hr. Steaming was then interrupted and both fibres showed no recovery. On steaming in the absence of tension, however, the deaminated fibre rapidly contracted, eventually (480 min.) being 45 % shorter than the original length, whereas the untreated fibre still remained

permanently elongated by 20%. Figs. 2 and 3 show the setting properties of our untreated and deaminated fibres: (*a*) on setting in boiling water for 3 hr. and (*b*) in 0·05M-borax for 30 min. at 100°,

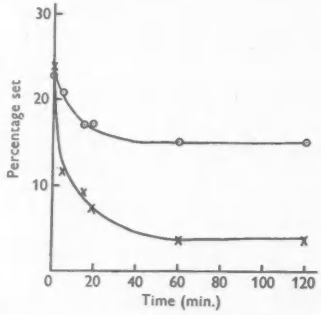

Fig. 2. The rate of release of set of untreated and deaminated wool fibres, in boiling water, after being set-extended in water at 100° for 3 hr. ⊙—⊙, untreated wool; ×—×, deaminated wool.

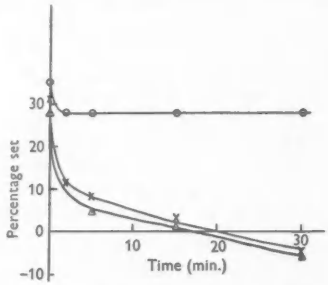

Fig. 3. The rate of release of set of untreated and deaminated wool fibres in boiling water after being set extended in boiling 0·5M borax for 30 min. ⊙—⊙, untreated wool; ×—×, deaminated wool; △—△, thrice deaminated wool.

respectively. It will be seen that our deaminated fibres retain more set than Speakman's (Cotswold) fibres. That this was not due to incomplete deamination was shown by the similarity in properties of once and thrice deaminated wool. It thus probably represents a difference between the wools used by Speakman and that used in the present investigation. Other differences of this kind have already been observed (Blackburn & Lindley, 1948).

The S *distribution of deaminated wools after boiling with buffers of* pH 8·2–10

The S distribution of deaminated wool which has been treated for different periods of time with boiling buffer solutions of pH 8·2, 9·2 and 10·0 is shown in Table 2. From these analytical results the loss of disulphide S, and sulphur loss are calculated as follows:

Loss of disulphide S = [thiol S + disulphide S in deaminated wool] − [thiol S + disulphide S in alkali-treated deaminated wool],

and

S loss = [total S − Br-oxidizable S + sulphate S in deaminated wool] − [total S − Br-oxidizable S + sulphate S in alkali-treated deaminated wool].

These values are shown in Table 3, together with the results of semi-quantitative lanthionine estimations carried out on some of the treated wools. The cysteic acid content, equivalent to about 0·2% S, remained unchanged after alkali treatment.

Compared with untreated wool (Cuthbertson & Phillips, 1945; Lindley & Phillips, 1945) the results are complicated in this case by the occurrence of substantial quantities of unidentified S in the fibre. The simplest explanation of the results, however, would seem to be that the cystine breaks down in the normal manner to give both lanthionine and combined α-aminoacrylic acid, whilst the unidentified S partly remains unchanged, and partly decomposes with complete loss of sulphur from the fibre. Thus the maximum lanthionine produced in deaminated wool (*c.* 0·8% S) plus half the unidentified S (0·6 ÷ 2 i.e. 0·3%) is equivalent to the 1·0–1·2% lanthionine S which is the maximum normally found for virgin wool after alkali treatment.

Table 4 gives the amino N content of some alkali-treated, deaminated wools. Subject to the reservations noted above, the results are interesting in showing an increase in amino N on alkali treatment in contrast to the decrease found in alkali-treated virgin wool by Cuthbertson & Phillips (1945). It would seem possible, therefore, that the peptide bonds in deaminated wool are less stable to alkali than is the case with untreated wool. Although the values are small they may, nevertheless, imply considerable changes in the average length of peptide chains and could thus greatly influence the physical properties of deaminated fibres.

The S *distribution of deaminated wool before and after treatment with dilute alkalis at room temperature*

A new batch of deaminated wool was used for these experiments. Its S distribution, before and after treatment with solutions of sodium hydroxide and barium hydroxide, is shown in Table 5, whilst Table 6 lists the derived S distributions of these wools.

These results contrast very sharply with those of Cuthbertson & Phillips (1945) on virgin wool, in

Table 2. *The* S *distribution of deaminated wool before and after treatment with boiling buffer solutions of* pH 8·2–10

Nature of treatment					
pH of buffer	Time of treatment (hr.)	Thiol + disulphide S (%)	Br-oxidizable S + SO_4 as S (%)	Methionine S (%)	Total S (%)
—	0	2·51	0·22	0·15	3·67
8·2	0·5	2·25	0·23	0·15	3·37
	1	2·06	0·29	0·15	3·34
	2	1·80	0·27	0·15	3·29
	3	1·51	0·27	0·15	3·11
	4	1·19	0·32	0·15	3·02
	5	1·08	0·34	0·15	2·89
9·18	0·25	1·68	0·70	0·15	3·28
	0·5	1·19	0·73	0·15	3·08
	1	0·72	0·65	0·15	2·69
	2	0·54	0·64	0·15	2·46
	4	0·54	0·33	0·15	2·17
10·0	0·25	1·52	0·60	0·15	3·07
	0·5	0·59	0·45	0·15	2·70
	1	0·45	0·45	0·15	2·53
	2	0·42	0·39	0·15	2·18
Before deamination		3·05	0·26	0·15	3·68

Table 3. *The non-disulphide* S, *disulphide* S *loss,* S *loss, and lanthionine content of deaminated wool after boiling with buffers of* pH 8·2–10

Nature of treatment						
pH of buffer	Time of treatment (hr.)	Non-disulphide S (%)	Increase in non-disulphide S on alkali treatment (%)	Disulphide S loss (%)	S loss (%)	Lanthionine S (%)
—	0	0·79	0	0	0	0·20
8·22	0·5	0·74	−0·05	0·26	0·31	0·35
	1	0·84	0·05	0·45	0·40	—
	2	1·07	0·17	0·71	0·43	—
	3	1·18	0·39	1·00	0·61	—
	4	1·37	0·58	1·32	0·75	—
	5	1·32	0·53	1·43	0·90	0·80
9·18	0·25	0·75	−0·04	0·83	0·87	0·45
	0·5	1·01	0·22	1·32	1·10	—
	1	1·17	0·38	1·79	1·48	—
	2	1·14	0·35	1·97	1·63	0·70
	4	1·15	0·36	1·97	1·61	—
10·0	0·25	0·80	0·01	0·99	0·98	0·40
	0·5	1·51	0·72	1·92	1·20	—
	1	1·48	0·69	2·06	1·32	—
	2	1·22	0·43	2·09	1·66	—
Before deamination		0·22	—	—	—	0·20

which lanthionine was rapidly formed. A slow breakdown, presumably of the Bergmann & Stather (1926) type, occurred in the baryta treatment, but was not evident at all in treatments with sodium hydroxide until the normality reached 1·0. The present results in deaminated wool, however, show that the major reaction occurring throughout is of the Bergmann & Stather type with complete loss of sulphur from the fibre, and the results with baryta suggest that this occurs not only with the cystine but also with the unidentified S.

Table 4. *The amino* N *content of deaminated wool before and after boiling with buffers of* pH 8·2–10

Nature of treatment		
pH of buffer	Time of treatment (hr.)	Amino N content (mg./g. wool)
—	0	1·27
8·22	3	2·30
9·18	2	2·30
10·0	0·5	2·13
Before deamination		3·05

Table 5. *The* S *distribution of deaminated wool before and after treatment with dilute solutions of* NaOH *and* $Ba(OH)_2$ *at room temperature*

Nature of treatment: Reagent	Time of treatment (hr.)	Thiol + disulphide S (%)	Br-oxidizable S + SO_4 as S (%)	Methionine S (%)	Total S (%)
—	0	2·52	0·47	0·15	3·67
0·1 N-NaOH	0·5	2·27	0·35	0·15	3·33
	2·5	1·82	0·46	0·15	3·15
	4·5	1·58	0·52	0·15	2·87
	7	1·23	0·46	0·15	2·68
	16·5	0·88	0·60	0·15	2·35
	24	0·82	0·51	0·15	2·24
	48	0·73	0·51	0·15	2·15
0·1 N-NaOH	1	2·11	0·63	0·15	3·15
0·5 N-NaOH	1	0·81	0·40	0·15	2·19
1·0 N-NaOH	1	0·44	0·32	0·15	1·68
0·385 N-$Ba(OH)_2$	0·25	1·47	0·32	0·15	2·40
	0·5	1·28	0·53	0·15	2·26
	1	0·82	0·58	0·15	2·25
	2	0·61	0·58	0·15	1·68
	4	0·43	0·34	0·15	1·08
	6	0·24	0·34	0·15	1·01

Table 6. *The non-disulphide* S, *disulphide* S *loss,* S *loss and lanthionine content of deaminated wool after treatment with dilute* NaOH *and* $Ba(OH)_2$ *solutions at room temperature*

Nature of treatment: Reagent	Time of treatment (hr.)	Non-disulphide S (%)	Increase in non-disulphide S on alkali treatment (%)	Disulphide S loss (%)	S loss (%)	Lanthionine S (%)
—	0	0·53	0	0	0	0·20
0·1 N-NaOH	0·5	0·56	0·03	0·25	0·22	0·30
	2·5	0·72	0·19	0·70	0·51	—
	4·5	0·62	0·09	0·94	0·85	—
	7	0·84	0·31	1·29	0·98	0·40
	16·5	0·72	0·19	1·64	1·45	—
	24	0·76	0·23	1·70	1·47	—
	48	0·76	0·23	1·79	1·56	—
0·1 N-NaOH	1	0·26	−0·27	0·41	0·68	—
0·5 N-NaOH	1	0·83	0·30	1·71	1·41	—
1·0 N-NaOH	1	0·77	0·24	2·08	1·84	0·70
0·385 N-$Ba(OH)_2$	0·25	0·46	−0·07	1·05	1·12	—
	0·5	0·30	−0·23	1·24	1·47	—
	1	0·70	0·17	1·70	1·53	—
	2	0·34	−0·19	1·91	2·10	—
	4	0·16	−0·37	2·09	2·46	—
	6	0·28	−0·25	2·27	2·53	—

DISCUSSION

The results obtained in the present investigation are important in providing another example of how the reactivity of a particular group (i.e. the disulphide bond of cystine) can be altered by modifications of other groups in the protein molecule (cf. Lindley & Phillips, 1947). The observation that lysine is absent from hydrolysates of deaminated wool, but that α-amino-ε-hydroxycaproic acid is present is of some importance in confirming accepted views on the mode of linkage of lysine. The spot corresponding to α-amino-ε-hydroxycaproic acid was only found in hydrolysates of deaminated wool and was not noted in alkali-treated, deaminated wool.

Nothing definite is known of the fate of arginine during deamination of wool. The first product might be citrulline which, according to Wada (1933), would form proline in boiling hydrochloric acid. Pure citrulline, however, under our conditions of hydrolysis (5 N-HCl, 24 hr.), was found by paper chromatography to be partially changed to ornithine, without formation of proline. No evidence of ornithine was found in the chromatograms of hydrolysates of

deaminated wool. Another possible product of deamination of arginine, α-amino-δ-hydroxyvaleric acid, is also stated by Sorensen (1905) to form proline on acid hydrolysis (conc. hydrochloric acid at 150°). Proline estimations were, therefore, carried out on hydrolysates of deaminated and untreated wool, using partition chromatography of acetamido acids on silica gel (Gordon, Martin & Synge, 1943; Tristram, 1946). No increase was found in the proline content of the hydrolysate of the deaminated wool. Thus the mechanism of breakdown of the combined arginine of the wool under these conditions remains obscure.

The results present a number of interesting points specifically concerned with the structure of wool. So far as is known at present, reagents which cause supercontraction of unstretched virgin wool (i.e. α-keratin) do so by fission of the disulphide bonds of cystine when cleavage is coupled with thermal agitation. The sole exception to this is potassium cyanide solution which Cuthbertson & Phillips (1945) found to produce lanthionine, but which Speakman (1936) claimed caused supercontraction of human hair. Numerous experiments in this laboratory, however, have failed to confirm Speakman's observation, and we have never obtained supercontraction of wool fibres or hair in cyanide solution. Deaminated wool would, however, seem to provide a genuine exception to this rule since it supercontracts readily in borax solution at 100° with formation of substantial quantities of lanthionine. Our deaminated fibres, for instance, supercontracted by 20–30% in 10 min. in boiling borax solution; reference to Tables 2 and 3 shows that after 15 min. at this pH and temperature the deaminated wool still contains 1·7% cystine S and 0·45% lanthionine S. Compared with deaminated wool, this represents a decrease of 0·55% S and, even compared to untreated wool, a loss of only 1·1% S present as covalent cross linkages. The work of Carter, Middlebrook & Phillips (1946) makes it improbable that such a degree of sulphur breakdown would lead to supercontraction.

The results on the setting of deaminated fibres are also difficult to reconcile with the chemical theory of setting. Thus, comparison of the present results with those on the action of alkalis on untreated wool (Lindley & Phillips, 1945) shows that the course of the reaction is not greatly influenced by the prior deamination treatment so far as boiling in weakly alkaline buffers are concerned, except to the extent that cystine S is destroyed by the deamination. This seems to be fairly constant and amounts to not more than 0·55% of cystine S which could form 0·27% of lanthionine S, or about 25% of the maximum amount of lanthionine produced in wool in mildly alkaline buffers at 100°. Whilst it is possible that this 25% may exert a decisive influence on the physical properties of the wool fibre, it seems probable in view of evidence from other sources (Blackburn & Lindley, 1948) that cystine reactivity does not play the decisive role in the mechanism of the setting of wool fibres in mildly alkaline buffers which has been attributed to it by Speakman (1936).

With the abandonment of the —S.NH— and lanthionine linkage as the important factors in the setting of wool in mildly alkaline buffers at 100°, the confirmation in the present work of Speakman's findings that deamination profoundly affects the setting properties raises another very important point with regard to wool structure. Thus Speakman & Hirst (1933) suggested that the basic and acid side-chains of wool were present as salt linkages between peptide chains, and the stability of the wool molecule has been regarded by Speakman as being primarily due to these salt linkages and covalent sulphur bonds. On this view, therefore, and accepting the suggestion that sulphur reactivity does not play a decisive part in setting under these conditions, it might be possible to regard set as being due to breakdown of the original salt linkages followed by reformation of new ones in the extended state. Such a hypothesis, however, implies that masking of the acidic side-chains would affect the setting properties of wool in the same way as modification of the basic side-chains by deamination. In point of fact, Blackburn & Lindley (1948) found that wool in which the free carboxyl groups were esterified by treatment with methylating agents behaved in exactly the opposite manner to deaminated wool, i.e. their setting properties were enhanced, and they showed a diminished tendency to supercontract in bisulphite solution. Apart from the possibility that the results on deaminated wool are due to some effect of nitrous acid on groups other than the basic side-chains, two further possibilities may be borne in mind: (1) the amino groups play a decisive part in set by forming a stable chemical cross link of unknown character, or (2) the basic and acidic side-chains of wool have entirely separate and distinct functions in the structure of wool, i.e. the salt-linkage hypothesis must be abandoned. The first hypothesis is rendered very unlikely by the observation of Cuthbertson & Phillips (1945) that the reaction of wool with nitrous acid is little affected by prior alkali treatment. The second suggestion is supported to some extent by the regularities in basic amino-acid composition found in keratins by Block (1939), whilst present (admittedly meagre) evidence suggests no such regularity of the dicarboxylic acid contents of keratins. With the apparent demolition of the long parallel peptide chain theory of wool by Farrant, Rees & Mercer (1947), the field would now seem to be clear for an entirely new synthesis of our knowledge of the structure of wool.

SUMMARY

1. The reaction of the sulphur of deaminated wool with alkalis has been studied.

2. It has been found that, excluding the cystine destroyed during deamination, the deaminated wool behaves very similarly to untreated wool with mildly alkaline buffers at 100°; in particular lanthionine is formed.

3. With stronger alkalis at room temperature, however, deaminated wool behaves in an entirely different manner from untreated wool and no lanthionine is formed.

4. An examination of some of the physical properties of deaminated wool has been made which has in general confirmed earlier observations of Speakman (1936).

5. Difficulties in reconciling the physical properties and the present chemical evidence with current views of wool structure are discussed.

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The Effects of Progressive Nutritional Hypoproteinaemia on the Extracellular-Fluid Phase and Plasma Colloid Osmotic Pressure in Rats

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Many theories have been advanced to explain the mechanism of oedema formation under conditions of malnutrition. It is widely accepted that the main factor concerned is a deficiency of dietary protein, which in turn results in the depletion of the proteins of the body and particularly of those of the blood plasma. According to Starling's (1895) hypothesis a decrease in the plasma proteins results in a fall of the plasma colloid osmotic pressure, a decreased ability to retain water in the capillaries, and a diffusion of fluid into the tissues. Support for the accepted theory has been provided both by animals fed on a protein-deficient diet (Kohman, 1920; Frisch, Mendel & Peters, 1929; Dicker, Heller & Hewer, 1946) and by human beings suffering from the so-called 'hunger oedema' (Gounelle, Bachet & Marche, 1942; Govaerts & Lequime, 1942; Mollison, 1946). However, Metcoff, Favour & Stare (1945)

claimed that feeding rats with a protein-deficient diet for several days produced no more than a slight decrease of the concentration of plasma proteins, whereas prolonged feeding with a somewhat less protein-deficient diet did not affect the plasma proteins. Furthermore, among clinical cases of 'hunger oedema', reported during and after the last war, a large proportion (seventeen in forty-three in the Govaerts & Lequime (1942) series) had a normal plasma protein level, with a correspondingly high colloid osmotic pressure. Finally, electrophoretic analysis of the plasma proteins of subjects suffering from experimental undernutrition did not show any hypoproteinaemia or a change in the albumin/globulin ratio (Keys, Taylor, Michelsen & Henschel, 1946), even though some of them had developed visible oedema. However, an increase of the thiocyanate space (and therefore very likely of the extracellular-fluid phase) was found in all these subjects whether they had developed oedema or not.

In view of these contradictory findings, the water and electrolyte distribution in skeletal muscle, in liver and in brain were studied in series of rats fed on a low-protein diet. The extracellular- and intracellular-water phases of these tissues were estimated.

METHODS

Experimental animals. Adult male albino rats weighing 270–320 g. were used.

Standard diet (*ST*). Control rats were given the following diet: casein 18, wheaten starch 29·5, hardened groundnut oil 9, dried yeast 11, salt mixture (Hawk & Bergeim, 1942) 3, cod-liver oil 2, water 27·5%. Each animal received daily *c.* 20 g. of this food, representing *c.* 60 kg.cal.

Vegetable low-protein diet (*turnip diet* = *TT*). The diet was modelled on that of Kohman (1920) and of Frisch *et al.* (1929) with fresh turnips taking the place of fresh carrots, and was the same as that used by Dicker *et al.* (1946). It had the following composition: fresh turnips 85·5, wheaten starch 7, hardened ground-nut oil 1·2, salt mixture 4·2, cod-liver oil 2·1%. In addition, each animal received a mixture containing 30 μg. aneurin, 50 μg. riboflavin and 1 mg. nicotinic acid. Fresh turnips contain *c.* 0·8% of protein.

The supply of this diet was not restricted. It was found that each animal ate *c.* 50 g. daily, which represented an intake of about 35–40 kg.cal. (The Medical Research Council War Memorandum (1945) was used for the calculation of calorific values.)

Low casein, high carbohydrate diet (*LCT*). The composition of this diet was as follows: casein 0·5, wheaten starch 54·0, hardened ground-nut oil 7·5%; salt mixture, cod-liver oil, vitamin B and tocopheryl acetate in the same amount as in the *TT* diet and water 31·7%.

Of this food 100 g. represented 303·5 kg.cal. This diet differed essentially from the *TT* diet in that the deficiency of calories due to the low protein content was compensated by an increased quantity of starch and fat. Each rat received about 20 g. of this mixture representing a daily intake of 60 kg.cal., comparable with that provided by the *ST* diet.

All rats had free access to water.

Procedure. All rats were fed on the standard diet for about 4 weeks, before being put on an experimental diet. Series of rats on *TT* diet were killed after 7, 14 or 42 days. The rats on *LCT* diet were killed after 70 days. Immediately after death, blood samples were taken and the stomach and the small intestine (up to the caecum) were removed and weighed. The two rectus abdominis muscles and two samples of the liver were excised and the brain removed and cut sagittally. Muscle, liver and brain samples were weighed in weighing bottles and analyzed for their Cl^-, Na, K and water content.

Analytical data collected. The following were determined for each rat: (*a*) the content of water, Cl^-, Na, K and N of heparinized plasma; (*b*) the content of water, Cl^-, Na and K of muscle samples; (*c*) the content of water, Cl^-, Na and K of liver samples; (*d*) the content of water, Cl^-, Na and K of brain samples.

Preparation of muscle, liver and brain tissues for Na *and* K *determination.* A known quantity of minced muscle, liver or brain was washed into a digestion tube with 10 ml. of distilled water and allowed to stand overnight. The mixture was then boiled for 20 min. The boiling was repeated twice at intervals of 3 hr. After the last boiling the mixture was allowed to settle, and the supernatant liquid was decanted into a 10 ml. volumetric flask. No filter was used and the device proposed by McCance & Shipp (1933) was adopted. The flask was cooled and its contents made up to the mark. It has been shown by Callow (1929) and McCance & Shipp (1933) that Na and K are quantitatively extracted from muscle tissue by this method. The same method was applied to the extraction of Na and of K from liver and brain samples.

Water determination in plasma and tissues. The water content of plasma, muscle, liver and brain was obtained by drying plasma and tissue samples at 104° for 48 hr.

Estimation of plasma colloid osmotic pressure. Keys & Taylor's (1935*a*) method and apparatus were used. Duplicate readings were made and corrected to 37°. The suspending and outer buffer fluid was made up as follows: KH_2PO_4 0·056 mol./l., KOH 0·0395 mol./l. The pH was 7·2.

Defatting of tissues. To express the water content of muscles as ml./100 g. of fat-free tissue, extraction of neutral fat was carried out according to Hastings & Eichelberger (1937). The same technique of fat extraction was applied to brain samples (Manery & Hastings, 1939).

Cl^- *determination.* Cl^- in plasma was determined by Whitehorn's (1921) method, using a 0·02N-NH_4CNS solution. For muscle, liver and brain tissue, the titration was carried out after acid digestion (Hastings & Eichelberger, 1937).

Na *determination.* Na in plasma and in tissue extracts was determined colorimetrically by the method of McCance & Shipp (1931).

K *determination.* K in plasma and in tissues was determined by Kramer & Tisdall's (1921) method as modified by McCance & Shipp (1933), using the empirical factor 0·12.

Protein determination in plasma. The plasma N content was estimated by a micro-Kjeldahl method. The digestion was carried out with SeO_2, and continued for 5 hr. after the clearing of the solution (Hoch & Marrack, 1945). The N was estimated colorimetrically, after nesslerization, and the protein concentration (in g./100 ml.) was calculated by multiplying the figures for N by 6·25.

The albumin fraction was estimated by the same method, the globulin having been precipitated by sodium sulphite (Campbell & Hanna, 1937).

Estimation of the extracellular-fluid phase of a tissue. The extracellular space of a tissue can be expressed either per 100 g. of fat-free solids or per 100 g. of fat-free wet tissue (Darrow, 1946; Fulton, 1946). The first method gives a better correlation between changes of the fat-free solids and their constituent N, P and K; on the other hand, the relationship between fat-free solids and tissue water, Cl^- and Na is not precise (Darrow, 1946). As changes of water, Cl^- and Na content of tissue were the main object of the present investigations, the second method was deliberately adopted.

The extracellular-fluid phase was expressed in terms of Cl^- (or Na) space. The method of calculating the Cl^- space was that of Eichelberger (1939):

$$\text{Extracellular water (ml./100 g. tissue)} = \frac{\text{mg. } Cl^-/100 \text{ g. tissue} \times \text{plasma water content}}{\text{mg. } Cl^-/100 \text{ g. plasma} \times 1{\cdot}04} \times 100.$$

To calculate the Na space a similar formula was used:

$$\text{Extracellular water (ml./100 g. tissue)} = \frac{\text{mg. Na/100 g. tissue} \times \text{plasma water content}}{\text{mg. Na/100 g. plasma} \times 0{\cdot}95} \times 100.$$

The cellular phase (g./100 g. tissue) = 100 – extracellular-fluid phase.

Calculation of the intracellular-fluid phase of a tissue. The intracellular-fluid phase was obtained by subtracting the figure for the extracellular-fluid phase from that of the total amount of water in 100 g. tissue.

Statistical treatment. Student's 't' test for small samples, as described by Fisher (1944), was used for estimation of the significance of means. The probability 'P' for 't' was obtained from Fisher & Yates's (1943) tables.

RESULTS

External appearance of rats on protein-deficient diets

Rats fed on *TT* diet for 7 and 14 days looked perfectly healthy, though their weight had decreased by 17 and 23 % respectively. However, rats fed on *TT* diet for 6 weeks looked emaciated. Their loss of body weight amounted to 33%. Their coats lost the normal smooth appearance, but the skin of the tail and feet was smooth. No ophthalmia or diarrhoea was noticed.

Rats fed on the low protein, high carbohydrate diet (*LCT*) lost 30% of their weight in 70 days. Nearly all of these animals showed large patches of alopecia, either on the back or on the head. They did not look emaciated.

Gross post-mortem findings in rats fed on protein-deficient diets

The post-mortem findings were as follows: nothing abnormal noted in rats fed on *TT* diet for 7 or 14 days. Oedema of the prepuce was observed in some of the rats which had received it for 6 weeks. No ascites was found in the present series. About two thirds of the animals had 'moist' tissues, while the remainder seemed to be abnormally 'dry'. None of the thoracic or abdominal organs showed any changes; in particular the liver appeared entirely normal. The latter findings conform to those made with a previous series of *TT* rats (Dicker *et al.* 1946). No ascites was found in *LCT* rats. All these animals had fatty deposits in the abdomen; their livers had a markedly fatty appearance.

Changes in plasma water concentration

There was no significant decrease in the plasma solids of rats fed on *TT* diet for 7 days as compared with control rats ($t = 1{\cdot}312$; $P = 0{\cdot}2$), nor was there any significant change in the protein concentration of their plasma (Table 1). But after 2 weeks on *TT* diet there was a marked increase in the plasma water accompanied by a decrease in the plasma protein concentration. Expressed in terms of plasma solids, there was a fall of 15%, while the protein level fell by 10% (Table 1).

The dilution of the plasma became more apparent the longer the period of malnutrition: after 6 weeks on *TT* diet the fall in the plasma solids and in the

Table 1. *Effect of protein-deficient diets on the total water content of plasma, muscle, liver and brain of rats*

(The values are means and standard errors. In parentheses, number of rats. All values for muscle and brain are expressed per 100 . fat-free tissue.)

Treatment of rats	Plasma protein concentration (g./100 ml.)	Plasma water content (g./100 ml.)	Muscle water content (g./100 g.)	Liver water content (g./100 g.)	Brain water content (g./100 g.)
Fed on a standard diet (*ST*)	7·06±0·090 (27)	91·4±0·13 (27)	75·7±0·32 (27)	71·4±0·31 (27)	83·6±0·24 (11)
Fed for 7 days on a protein-deficient diet (*TT*)	6·75±0·331 (9)	91·8±0·15 (9)	75·0±0·51 (9)	72·6±0·48 (9)	82·7±0·21 (9)
Fed for 14 days on a protein-deficient diet (*TT*)	6·46±0·213 (9)	92·7±0·21 (9)	77·0±0·23 (9)	72·3±0·75 (9)	82·2±0·12 (9)
Fed for 42 days on a protein-deficient diet (*TT*)	5·02±0·137 (29)	93·2±0·16 (29)	77·2±0·28 (30)	74·9±0·39 (30)	83·3±0·41 (12)
Fed for 70 days on a low casein diet (*LCT*)	4·91±0·186 (6)	93·6±0·16 (6)	74·2±0·55 (6)	68·3±1·70 (6)	82·9±0·70 (6)

concentration of proteins was 21 and 27% respectively (Table 1).

The same phenomenon was noticed in rats fed on a low casein diet (*LCT*) for a longer period. In spite of the gross accumulation of fat in the body, plasma solids and proteins fell by nearly 30%.

Changes in the water content of tissues

No changes in the total water content of muscles were found in animals which had been fed on *TT* diet for 7 days as compared with control rats ($t = 1{\cdot}136$, $P > 0{\cdot}2$) (Table 1). However, the liver already showed a slight increase of its total water content ($t = 2{\cdot}010$, $P < 0{\cdot}1$).

Table 1 shows changes in total water content of muscles, liver and brain in rats fed on *TT* diet for 14 and 42 days.

When comparing total water content of muscles and liver of rats after 42 days of *TT* diet and after 70 days on *LCT* diet, it will be seen that the latter animals had significantly less water in their tissues than the former ($t = 4{\cdot}411$, $P < 0{\cdot}001$, and $t = 5{\cdot}500$, $P < 0{\cdot}001$); the water content of muscle and liver samples from *LCT* rats was actually comparable with that of control rats.

Changes in the ionic composition of plasma and tissues

Plasma. Changes in the chloride, sodium and potassium concentration of plasma in *TT* rats during varying periods of observation are shown in Table 2. Comparing these changes with those of the plasma water (Table 1) in the same rats at corresponding periods, it will be seen that changes in the ionic pattern are independent of those of plasma water content, i.e. occasional decreases in plasma sodium (after 7 days) or chloride concentration (after 14 days) are not the result of a dilution of the plasma.

The independence of the concentration of the ions in plasma from that of the plasma water emerges even more clearly when the changes in the potassium level of the plasma are considered: the potassium level in the plasma of *TT* rats rose during the whole period of observation, in spite of the rising dilution of the plasma. In the *LCT* rats, it remained unaffected.

Tissues. It would be tempting to assume that the fact that chloride and sodium were not diluted by an increased amount of water in plasma was the result of an influx of these ions from the tissues. However, Table 2 shows that so far as chloride and sodium are concerned, their concentration increased significantly in muscle and in liver ($t = 7{\cdot}529$, $P < 0{\cdot}001$ and $t = 3{\cdot}231$, $P < 0{\cdot}001$). This was already apparent in rats fed on a turnip diet for 7 days only.

Comparing Tables 1 and 2, it will be seen that, as in plasma, changes in the chloride and sodium concentration of muscle and liver were independent of the concurrent changes in their water content. But in contrast to that of muscle and liver tissues, the

Table 2. *Ionic composition of plasma, muscle, liver and brain in normal and hypoproteinaemic rats*

(The values are means and standard errors. In parentheses, number of rats. All values for muscle and brain are expressed per 100 g. fat-free tissue.)

Treatment of rats	Cl^- (m-equiv./l. water)	Na (m-equiv./l. water)	K (m-equiv./l. water)	Cl^- (m-equiv./l. water)	Na m-equiv./l. water)	K (m-equiv./l. water)
		Plasma			Muscle	
Fed on a standard diet (*ST*)	83·2±2·34 (27)	128·3±7·05 (18)	5·6±0·29 (17)	20·2±0·47 (17)	28·8±1·48 (17)	126·2±7·73 (17)
Fed for 7 days on a protein-deficient diet (*TT*)	84·6±3·08 (9)	119·3±7·53 (9)	5·0±0·27 (9)	27·8±0·82 (9)	35·2±3·59 (9)	107·6±5·78 (9)
Fed for 14 days on a protein-deficient diet (*TT*)	83·7±4·90 (9)	124·0±6·94 (9)	6·0±0·25 (9)	31·5±1·01 (9)	46·5±1·42 (9)	112·4±7·14 (9)
Fed for 42 days on a protein-deficient diet (*TT*)	85·9±2·77 (29)	108·3±2·12 (11)	7·1±0·58 (11)	46·7±2·44 (29)	69·5±4·22 (11)	96·6±3·84 (11)
Fed for 70 days on a low casein diet (*LCT*)	81·0±2·03 (6)	120·6±2·46 (6)	5·1±0·20 (6)	37·2±2·09 (6)	60·0±4·60 (6)	107·9±5·30 (6)
		Liver			Brain	
Fed on a standard diet (*ST*)	33·6±2·80 (11)	31·0±2·09 (10)	112·8±7·63 (10)	42·7±2·89 (9)	49·0±3·56 (10)	124·0±10·51 (10)
Fed for 7 days on a protein-deficient diet (*TT*)	41·4±2·99 (9)	50·7±5·61 (9)	103·7±4·87 (9)	41·1±2·69 (9)	52·3±4·37 (9)	129·5±5·26 (9)
Fed for 14 days on a protein-deficient diet (*TT*)	40·2±0·66 (9)	60·6±3·00 (9)	97·8±8·13 (9)	38·0±1·75 (9)	56·5±2·42 (9)	118·6±3·08 (9)
Fed for 42 days on a protein-deficient diet (*TT*)	36·4±2·55 (11)	48·7±3·24 (11)	93·8±3·61 (11)	39·1±3·27 (11)	59·5±3·25 (11)	114·4±4·44 (11)
Fed for 70 days on a low casein diet (*LCT*)	49·0±2·69 (6)	57·2±6·21 (6)	120·0±6·63 (6)	41·7±1·25 (6)	58·4±3·36 (6)	109·7±8·89 (6)

ionic composition of brain samples remained constant during the whole period of observation (Table 2).

Such changes in the water content and in the ionic composition of plasma and tissues made it likely that variations had occurred in the amount of extracellular water in muscle and liver.

Extracellular-fluid phase of tissues

In normal rats fed on a standard diet (*ST*) the chloride space of muscle, liver or brain was comparable with, and almost equal to, the sodium space (Table 3). It will also be seen from Table 3 that the extracellular-fluid phase was of characteristic magnitude in each of the tissues investigated and showed little variation in individual normal animals.

However, in *TT* rats, the extracellular-fluid phase of muscle and liver was increased. This increase was already significant after 7 days: it amounted to 44 % in muscles and to 50 % in liver. It increased steadily. The increase after 6 weeks amounted to 163 and 55% of the normal.

In Table 3 all data obtained for rats kept on *TT* diet for 42 days were pooled, irrespective of the fact that about one third of these animals had particularly dry and thin muscles. But the individual figures obtained from analysis of muscle samples of such 'dry' animals showed a marked increase in the extracellular-fluid phase, amounting to nearly 140%. Whether 'dry' or 'wet', all the *TT* rats had therefore a marked 'oedema' of their tissues. It follows that there was no essential difference between the two types of protein-deficient animals (Dicker, 1947).

In rats fed on a low casein, high carbohydrate diet (*LCT*) the extracellular-fluid phase of muscle and liver was also increased. The increase amounted to 120 and 70 % respectively. It is thus clear that the increased extracellular-fluid phase of *TT* and *LCT* rats was not the result of a deficiency of calories.

It could thus be shown that there were signs of early oedema in the tissues of rats fed on a protein-deficient diet. This increase of the extracellular-fluid space developed in spite of normal plasma protein

Table 3. *Extracellular- and intracellular-fluid phases (chloride and sodium) of tissues of normal and hypoproteinaemic rats*

(The values are means and standard errors. In parentheses, number of rats. All values for muscle and brain are expressed per 100 g. fat-free tissue.)

Treatment of rats	Extracellular-fluid phase (ml./100 g.)		Intracellular-fluid phase (ml./100 g.)	
	Cl^-	Na	Cl^-	Na
		Muscle		
Fed on a standard diet (*ST*)	16·7±0·50 (27)	16·8±1·03 (17)	59·0±0·50 (27)	58·8±1·11 (17)
Fed for 7 days on a protein-deficient diet (*TT*)	24·1±1·54 (9)	28·1±3·68 (9)	50·8±1·74 (9)	46·9±3·94 (9)
Fed for 14 days on a protein-deficient diet (*TT*)	32·3±2·78 (9)	30·1±1·89 (9)	44·7±2·72 (9)	47·0±1·83 (9)
Fed for 42 days on a protein-deficient diet (*TT*)	44·8±1·90 (29)	43·5±2·08 (11)	32·4±1·64 (29)	33·6±0·70 (11)
Fed for 70 days on a low casein diet (*LCT*)	36·9±3·63 (6)	36·8±2·01 (6)	37·3±3·65 (6)	37·4±2·27 (6)
		Liver		
Fed on a standard diet (*ST*)	22·6±2·50 (11)	21·3±1·55 (10)	48·5±2·05 (11)	50·1±2·03 (10)
Fed for 7 days on a protein-deficient diet (*TT*)	34·3±2·64 (9)	35·2±2·23 (9)	38·6±2·97 (9)	37·6±2·94 (9)
Fed for 14 days on a protein-deficient diet (*TT*)	38·1±2·56 (9)	34·8±2·73 (9)	34·2±2·57 (9)	48·0±2·74 (9)
Fed for 42 days on a protein-deficient diet (*TT*)	35·4±2·66 (11)	32·6±1·91 (11)	39·5±2·52 (11)	42·3±2·14 (11)
Fed for 70 days on a low casein diet (*LCT*)	39·0±2·03 (6)	35·4±3·22 (6)	29·3±2·07 (6)	32·8±4·09 (6)
		Brain		
Fed on a standard diet (*ST*)	38·6±3·31 (9)	40·1±3·00 (10)	44·9±3·24 (9)	43·5±2·89 (10)
Fed for 7 days on a protein-deficient diet (*TT*)	38·7±2·62 (9)	43·0±4·82 (9)	43·2±2·81 (9)	39·5±4·90 (9)
Fed for 14 days on a protein-deficient diet (*TT*)	40·6±2·24 (9)	38·1±2·24 (9)	41·8±2·18 (9)	44·0±2·29 (9)
Fed for 42 days on a protein-deficient diet (*TT*)	40·4±3·31 (11)	43·9±2·71 (11)	42·9±3·60 (11)	39·4±2·37 (11)
Fed for 70 days on a low casein diet (*LCT*)	41·0±1·51 (6)	38·4±2·14 (6)	41·9±1·40 (6)	44·9±2·31 (6)

concentrations. It could be shown further that the extracellular-fluid space of tissues (muscle and liver) increased whether the protein-deficient diet was, or was not, lacking in calories.

The intracellular-fluid phase of tissues

Can the increased extracellular-fluid phase observed in the different series of *TT* and *LCT* rats be explained by changes in the total muscle water? Comparing the data of Table 1 and Table 3, it could be concluded that this was not the case: in animals fed on *TT* diet for 7 days the extracellular-fluid space increased without any changes in the total muscle water. Even in rats fed on *TT* diet for 14 or 42 days the increase in the total water of the tissues (Table 1) was far less pronounced than that of the extracellular-fluid space (Table 3). It follows that tissue oedema developed concurrently with a marked loss of intracellular water. This is even clearer with *LCT* rats (Table 3).

It is thus clear that the retention of tissue water resulting from a lack of protein in the food did not produce a swelling of the tissue cells; on the contrary, protein deficiency led to a decrease of intracellular water. This decrease became the more obvious the more the extracellular-fluid space increased.

Colloid osmotic pressure of plasma

It has not been shown so far whether the decreases in the plasma protein concentration observed led to corresponding decreases in plasma colloid osmotic pressure. Values of $18{\cdot}9 \pm 0{\cdot}12$ cm. water, corrected to a temperature of 37°, were obtained for normal rat plasma. These values were somewhat lower than those found by Landis (1930), but this difference may be accounted for by the difference in pH and in electrolyte concentration of the buffer solution producing different Donnan effects (Marrack & Hewitt, 1927; Keys & Taylor, 1935*a*).

Table 4 gives figures for the colloid osmotic pressure of plasma samples from normal and hypoproteinaemic rats.

Dilutions of plasma of normal rats were made by adding varying amounts of the buffer solution. Fig. 1 shows that the colloid osmotic pressure of progressive dilutions of plasma of normal rats yielded values which form a straight line when plotted against the plasma protein concentration.

Table 4. *Colloid osmotic pressure of plasma samples from normal and hypoproteinaemic rats*

	Plasma proteins (Kjeldahl) (g./100 ml.)	Albumin/globulin ratio (A/G)	Plasma colloid osmotic pressure (cm. water)
Normal rats	7·48	1·20	19·8
	7·48	1·10	19·6
	7·25	1·20	20·0
	7·04	1·17	19·4
	6·75	1·07	19·2
Mean and S.E.	7·20±0·100	1·15±0·023	19·6±0·12
Rats fed on a protein-deficient diet (*TT*) for 7 days	7·18	0·98	19·1
	7·08	1·00	19·6
	7·00	1·26	19·4
	6·87	0·95	18·5
Mean and S.E.	7·03±0·057	1·05±0·031	19·2±0·19
Rats fed on a protein-deficient diet (*TT*) for 14 days	6·63	0·83	17·7
	6·55	0·90	17·3
	6·08	0·80	16·3
	6·07	0·70	15·4
	6·00	0·70	14·6
Mean and S.E.	6·22±0·121	0·78±0·011	16·3±0·51
Rats fed on a protein-deficient diet (*TT*) for 42 days	5·94	0·60	12·8
	5·94	0·60	10·8
	5·45	0·68	13·7
	5·27	0·50	8·6
	5·16	0·60	10·0
	5·10	0·62	11·7
	4·52	0·50	5·5
	3·95	0·50	8·6
Mean and S.E.	5·16±0·223	0·57±0·007	10·2±0·87
Rats fed on a protein-deficient diet (*LCT*) for 70 days	5·14	0·56	10·6
	4·37	0·67	10·8
	4·37	0·68	12·7
	3·91	0·68	11·3
	3·86	0·59	7·8
Mean and S.E.	4·33±0·205	0·63±0·023	10·6±0·71

This relationship applied only for dilutions of the plasma up to 50 % of the initial concentration, confirming Verney's (1926) findings on human plasma.

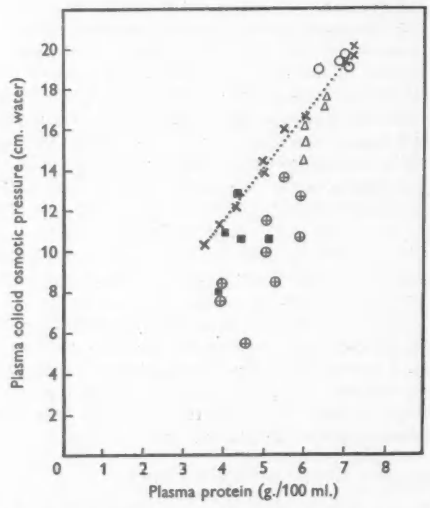

Fig. 1. Relation between the plasma colloid osmotic pressure and plasma protein concentration. × = normal rats; ○ = rats fed for 7 days on a vegetable protein-deficient diet; △ = rats fed for 14 days on a vegetable protein-deficient diet; ⊕ = rats fed for 42 days on a vegetable protein-deficient diet; ■ = rats fed for 70 days on a low casein diet. All the values for colloid osmotic pressure are corrected to 37°.

Comparing the values for the colloid osmotic pressure of diluted normal plasmas with those of samples from hypoproteinaemic rats, it will be seen (Fig. 1) that, at the same concentration of plasma proteins, the colloid osmotic pressure of plasma of rats fed on *TT* diet for 42 days and of *LCT* rats was lower than that of the normal animals. This decrease in the colloid osmotic pressure per g. plasma protein in animals fed on a low protein diet was progressive: plasmas of rats fed on *TT* diet for 7 days did not show a significant fall in the colloid osmotic pressure ($t = 1{\cdot}581$, $P > 0{\cdot}1$), but after 14 days the fall of colloid osmotic pressure was greater than that produced by a corresponding dilution of plasma of normal rats. It is thus clear that the low figures obtained for the osmotic pressure of plasma proteins in rats fed on protein-deficient diets could not be the result of a simple dilution of the blood of these animals.

The decrease in the colloid osmotic pressure observed in the plasma of rats fed on protein-deficient diets was closely correlated with the fall in the concentration of albumin; that of the globulin remained more or less unaffected. In other words, the colloid osmotic pressure per g. of total plasma proteins was directly proportional to the albumin/globulin (A/G) ratio (Table 4). However, in a few cases plasma samples with the same concentration of proteins had a widely different colloid osmotic pressure, e.g. two plasma samples with protein concentrations of 5·9 g./100 ml. gave values for the colloid osmotic pressure of 12·8 and 10·8 cm. water. The difference in these instances could not be accounted for by changes in the albumin/globulin ratio (A/G = 0·60 in both cases). Two other samples with the same A/G ratio (0·50) had a colloid osmotic pressure of 8·6 and 5·5 cm. water respectively. It is thus clear that in these particular cases, changes in the A/G ratio did not account for the low colloid osmotic pressure. It thus seems likely that the plasma proteins of some of the hypoproteinaemic animals underwent other changes in chemical or physical composition besides the decrease in the albumin fraction.

Finally, is there a relation between the colloid osmotic pressure of plasma and the occurrence of tissue oedema? Comparing the figures in Table 3 and Table 4 it will be seen that the increase in the extracellular-fluid space in rats fed on *TT* diet for 7 days occurred in animals with normal plasma protein concentration, normal A/G ratio and normal colloid osmotic pressure. Furthermore, no correlation could be found between the size of the extracellular-fluid space and the plasma colloid osmotic pressure.

DISCUSSION

There is ample evidence that chloride and sodium in rat and dog muscle are mainly extracellular (Fenn, 1936; Harrison, Darrow & Yannet, 1936; Hastings & Eichelberger, 1937; Manery & Bale, 1941). Further proof for the validity of this statement comes from the work of Manery & Haege (1941), Hevesy & Rebbe (1940) and Cuthbertson & Greenberg (1945) with radioactive isotopes ^{38}Cl and ^{28}Na (or ^{22}Na). These authors have shown that these isotopes were extracellular and that their distribution was the same. Finally, there is a satisfactory agreement between values for chloride space in muscle and liver and the extracellular space as determined microscopically in frozen sections of these tissues (Höber, 1947; Truax, 1939). At the same time it must be remembered that chloride and sodium spaces are likely to be somewhat larger than the true extracellular-fluid space, even in normal animals (Fulton, 1946), and that the possibility obtains that pathological conditions may produce further alteration of cell permeability. The chloride and sodium spaces may, therefore, not be quite identical with the true extracellular space. The term extracellular-fluid space in this paper must be understood to refer to

the apparent volume distribution of chloride and sodium.

In the present investigation it could be shown that, for muscles of normal rats, there is good agreement between the values of the extracellular-fluid phase, whether expressed in terms of chloride or of sodium space ($t=0{\cdot}113$, $P>0{\cdot}9$). The values compare favourably with those found by means of radioactive isotopes in the muscle of rats (Cuthbertson & Greenberg, 1945) and in dogs (Eichelberger, 1941). In the liver also it could be shown that the values for extracellular-fluid phase, whether expressed as chloride or as sodium space, were comparable ($t=0{\cdot}429$, $P>0{\cdot}6$). They were of the same magnitude as that of chloride space of liver found in dogs (Eichelberger, 1941).

When defining the intracellular-fluid phase it must be remembered that the amount of water inside the cell is correlated with the amount of potassium and that, under normal conditions, there is no shift of bases across the cell membrane (Peters, 1944). There is a reciprocal relationship between the extracellular sodium and the intracellular potassium; in other words, the amount of intracellular potassium, and intracellular water, is indirectly influenced by the concentration of extracellular sodium (Darrow, 1945).

It could be shown in the present investigation that the extracellular-fluid phase of muscle of normal adult rats was fairly constant. However, in rats fed on a protein-deficient diet for a short period only (7 days) the extracellular-fluid space of muscle increased significantly. This onset of tissue oedema occurred without significant changes in the plasma water content, the plasma protein concentration or the osmotic pressure of the plasma proteins. These findings agree with those of Keys *et al.* (1946) in human beings, receiving experimentally a low-protein diet, in whom an increase of the thiocyanate space (i.e. of the extracellular space) could be demonstrated at a time when the plasma protein concentrations, the plasma electrophoretic pattern and the plasma colloid osmotic pressure were still normal. It thus seems difficult to attribute the early onset of the oedema to a fall of the colloid osmotic pressure (Starling's hypothesis), but it might still be due to an increase in the capillary pressure (Pappenheimer & Soto-Riviera, 1947). It was not possible to estimate the venous pressure in the present series of rats; but in human beings Keys *et al.* (1946) showed that the venous blood pressure had fallen by nearly 50 % of the normal value.

The cause of the early increase in the extracellular-fluid phase must thus be sought elsewhere. The rats fed on a vegetable low-protein diet were lacking in both proteins and calories. There are thus good reasons to assume that a cellular breakdown was accelerated. Hence the early tissue oedema may have been due to an accumulation of waste products: if of small molecular weight they would exert a high osmotic pressure. The same process may also involve liberation of potassium ions, which in normal tissue were osmotically inactive. A higher osmotic pressure within the cell would result in an increased concentration of chloride and sodium in the extracellular-fluid space in an attempt to equalize osmotic pressure. To be able to maintain a balance between forces engaged in such a dynamic equilibrium the cells would extrude their waste products and with them part of their potassium, and ultimately would lose some of the intracellular water. This assumption would agree with the findings of Gamble, Ross & Tisdall (1923), who showed that starvation induces a loss of potassium from the body corresponding to the potassium content of the muscle tissue lost.

The data in Table 3 show that the increase of the extracellular-fluid phase was significantly greater than the decrease of the intracellular water. This can only mean that the increased extracellular-fluid phase was made up mainly by water from the plasma. After 7 days on a low protein, low calorie diet (*TT*) the plasma water content and its protein concentration were normal (Table 1). This is in agreement with findings in dogs fasting for a period of 4–6 days (Kerr, Hurwitz & Whipple, 1918). The unchanged level of the plasma proteins in rats fed for 7 days on *TT* diet might be explained by changes in the plasma volume masking the loss of plasma proteins, resulting from a state of semi-starvation. It would also agree with the findings of Metcoff *et al.* (1945) in rats kept on a protein-deficient diet.

At a later stage, in rats fed on *TT* diet for 14 days, the concentration of plasma proteins was decreased, though the A/G ratio was not significantly altered; and the colloid osmotic pressure was somewhat lower than in controls (Table 4; Fig. 1). Though small, these changes in the colloid osmotic pressure of plasma may have been sufficient to produce an increase of the extracellular-fluid phase, provided that there was no concurrent drop in the capillary pressure.

In rats fed on *TT* or *LCT* diet for several weeks the colloid osmotic pressure of the plasma proteins was markedly lower than that of the controls. In some cases it was even lower than that expected for the corresponding amount of plasma proteins. Such discrepancies between the plasma protein concentration and the expected colloid osmotic pressure have been reported in patients suffering from malnutrition (Govaerts & Lequime, 1942) and in young men exercised to exhaustion where, in spite of a marked increase (averaging 12·5 %) in serum protein concentration, the colloid osmotic pressure decreased (Keys & Taylor, 1935*b*). In rats fed on a protein-deficient diet (*TT* or *LCT*) the globulin fraction did not seem to be seriously affected; it is thus likely that the abnormally low colloid osmotic

pressure encountered in some cases was the result of changes in the albumin fraction. Two explanations of this phenomenon may be proposed:

(*a*) The chemical analysis of plasma proteins after precipitation of globulins by sodium sulphite may have given too high values for the albumin fraction (Cohn & Wolfson, 1947). This hypothesis receives further support from the work of Zeldis, Alling, McCoord & Kulka (1945). Comparing data obtained by chemical analysis and by electrophoretic methods these authors have shown that in dogs fed on a protein-deficient diet for 12 weeks the degree of depletion of 'electrophoretic albumin' is considerably greater than that of the chemical albumin fraction.

(*b*) Changes in the molecular dimension of the proteins or in their ionization, or in both, provide the basis for another interpretation. For any change in the colloid osmotic pressure to be due to changes other than in the molecular dimension, there must be changes in the ionization of the proteins or changes in the concentration of the crystalloids of the medium (Keys & Taylor, 1935*b*). Both pH and ionic strength, inside and outside the collodion membrane of the osmometer, remained constant in the present experiments. This makes any appreciable change in the magnitude of the Donnan effect unlikely. Furthermore, it has been shown that the colloid osmotic pressure of blood plasma is little affected by changes in pH (Marrack & Hewitt, 1927) or by moderate changes in salt concentration (Meyer, 1932).

It would seem that there are two stages in the production of oedema in tissue resulting from feeding rats on a protein-deficient diet. It could be shown that a significant increase of the extracellular-fluid phase of muscles occurred very early, without any significant changes in the water content or the protein concentration of plasma; no changes in the colloid osmotic pressure of plasma were observed at this stage. It must therefore be concluded that the onset of oedema formation in malnutrition is independent of changes in the colloid osmotic pressure of plasma. At a later stage further increases of the extracellular-fluid phase coincided with the progressive fall in the colloid osmotic pressure of the plasma. However, even then no clear correlation could be found between the degree of hypoproteinaemia, the fall of the colloid osmotic pressure of plasma and the magnitude of the extracellular-fluid phase of tissues.

SUMMARY

1. The total water content of plasma, of skeletal muscle, of liver and of brain was estimated in normal rats and in rats fed for periods of 7, 14 and 42 days on a protein-deficient diet. Extracellular- and intracellular-fluid phases of these tissues were calculated by means of chloride and sodium estimations.

2. The calculated values for the extracellular-fluid phase of these tissues agreed well, whether expressed as chloride or as sodium space.

3. In normal rats fed on a standard diet (*ST*) the extracellular-fluid phase of muscle, liver and brain, expressed as chloride space, amounted to $16{\cdot}7 \pm 0{\cdot}50$; $22{\cdot}6 \pm 2{\cdot}50$ and $38{\cdot}6 \pm 3{\cdot}31$ ml./100 g. fat-free tissue respectively.

4. In rats fed for 7 days on a vegetable protein-deficient diet (*TT*) the water content of plasma and of tissues, the plasma protein concentration and the plasma colloid osmotic pressure remained unchanged. In spite of this the extracellular-fluid phase of skeletal muscles and of liver had significantly increased, showing that there was tissue oedema with normal plasma protein concentration and normal colloid osmotic pressure.

5. In rats fed for 14 days on a vegetable protein-deficient diet (*TT*) there was a significant increase in the water content of the plasma, the muscles and the liver, with a decrease in the plasma protein concentration and a slight but significant decrease in the colloid osmotic pressure of plasma. The extracellular-fluid phase of the muscles was significantly greater than that of rats fed for 7 days on *TT* diet.

6. Comparing data obtained for rats fed for 42 days on a low-protein low-calorie diet (*TT*) with those for animals fed for 70 days on a diet equally low in protein but yielding an adequate supply of calories (*LCT*) it could be shown that the extracellular-fluid phases of muscle and liver were markedly increased, and the concentration of proteins in plasma significantly decreased in both series.

7. The mean colloid osmotic pressure of normal rat plasma amounted at 37° to $19{\cdot}6 \pm 0{\cdot}12$ cm. water; that of rats fed on *TT* diet for 7 days was $19{\cdot}2 \pm 0{\cdot}19$ cm., that of rats fed on *TT* diet for 14 days was $16{\cdot}3 \pm 0{\cdot}51$ cm., that of animals fed on *TT* diet for 42 days was $10{\cdot}2 \pm 0{\cdot}87$ cm., and that of animals fed on *LCT* diet for 70 days amounted to $10{\cdot}6 \pm 0{\cdot}71$ cm. water.

8. The colloid osmotic pressure of plasma of the three latter groups was correlated with the albumin/globulin (A/G) ratio of plasma, but not with the nitrogen concentration of the plasma samples.

9. In the cases of a few rats fed for 42 days on *TT* diet and for 70 days on *LCT* diet, the colloid osmotic pressure of plasma samples was lower than that expected from either the A/G ratio or the plasma nitrogen concentration.

10. No correlation could be found between the increase of the extracellular-fluid phase of tissues and the decrease of the colloid osmotic pressure of the plasma.

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Changes in the Extracellular- and Intracellular-Fluid Phases of Tissues during Water Diuresis in Normal and Hypoproteinaemic Rats

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It has been shown in a previous paper (Dicker, Heller & Hewer, 1946) that the urinary excretion of administered water by protein-deficient rats is delayed, and that the total amount of water excreted in 3 hr. is lower than that of normal animals. There would seem to be at least three likely explanations for the abnormal and delayed diuretic response observed in these rats: (*a*) a slower rate of water absorption from the alimentary canal; (*b*) an increased 'preparedness' of extrarenal tissues to retain water, resulting in a decrease of the plasma water load; (*c*) a failure of the renal tubules to reduce the rate of water reabsorption as a response to the increased water load; or a combination of these factors.

To investigate these possibilities, the rate of water absorption from the gastro-intestinal tract was estimated according to Heller & Smirk (1932), and the partition of absorbed water between the extracellular-fluid phases of tissues was determined.

METHODS

Experimental animals. Adult male albino rats weighing 265–310 g. were used.

Diets. The standard diet (*ST*) and the vegetable low-protein diet (*TT*) conformed to the description given in the previous paper (Dicker, 1948*a*).

Procedure. Normal rats were fed on a standard diet (*ST*) for several weeks. Food, but not water, was withheld 24 hr. before the diuresis experiments. The animals were then given water to the extent of 5% of their body weight by stomach tube, and were killed 15, 30, 45, 60, 75, 90 and 120 min. after the administration.

Another series of rats was fed on diet *TT*. After 6 weeks on this diet, food was withheld for 24 hr. and water to the extent of 5% of their body weight was given. They were killed 15, 30, 45, 60 and 90 min. after the administration.

Immediately before they were due to be killed, the rats were anaesthetized and blood collected from the carotid and jugular was mixed with heparin. Immediately after death the cardia and the distal end of the small intestine (excluding the caecum) were ligatured. The alimentary canal was weighed with its contents. Muscle, liver and brain samples were obtained and the amount of water, Cl^-, Na and K in these tissues estimated.

Chemical methods. Cl^-, Na, K and water estimations in plasma and tissue samples conformed to those described previously (Dicker, 1948*a*). Water content was calculated for fat-free tissues.

Protein concentration in plasma was estimated by two different methods: (*a*) a micro-Kjeldahl method, (*b*) the copper sulphate method for measuring plasma specific gravities (Phillips, Van Slyke, Dole, Emerson, Hamilton & Archibald, 1945). The formula used for the calculation of the plasma protein concentration was that proposed by Hoch & Marrack (1945). The two methods gave results which were not significantly different (Dicker, 1948*b*).

Estimation of the extracellular- and intracellular-fluid phase of tissues. Extracellular and intracellular tissue-fluid phases were calculated from estimation of Cl^- and Na spaces, as in the previous paper (Dicker, 1948*a*), where the identity of these spaces with the true extracellular-fluid phase has also been discussed.

Sampling of rats. The rats were killed in groups consisting of animals with varying absorption times. For statistical treatment of the data, results obtained for animals with identical absorption times were pooled.

Statistical treatment. Results are given as mean and standard errors. Student's '*t*' test for small sample method was used for the significance of means and calculated according to Mainland (1938). *P* values for *t* were obtained from Fisher & Yates's (1943) tables.

RESULTS

Gross post-mortem observation

Rats fed on diet *TT* for 6 weeks had lost about 35% of their body weight; the concentration of proteins in plasma had equally decreased by 35%. No ascites or free fluid was found in any of these animals, even after administration of water, but nearly all those that had received water had abnormally 'wet' tissues. The perirenal and retroperitoneal connective tissues were particularly oedematous, and had a gelatinous appearance. Pressure on these tissues released a substantial quantity of water (up to 1·5 ml.).

In all other respects, these series of protein-deficient animals were comparable with those described in the previous paper (Dicker, 1948*a*).

Water absorption

Fig. 1 shows the rate of water absorption from the alimentary canal in normal and hypoproteinaemic rats. The percentage of body weight of the alimentary canal in normal control rats was 3·6 as compared with 5·7 in controls fed on *TT* diet. In order to render the two curves more easily comparable 2·1 (i.e. the difference between the weights of the alimentary canal in the two series) was deducted from the mean values obtained in the protein-deficient series (Fig. 1).

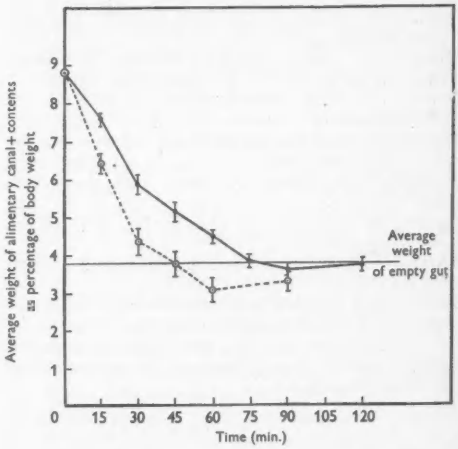

Fig. 1. Average water-absorption curves of rats: (*a*) •—• in normal rats; (*b*) ⊙– – –⊙ in hypoproteinaemic rats. The average weight of the empty gastro-intestinal tract was 3·6% of the body weight in normal control rats as compared with 5·7% of the body weight in controls fed on *TT* diet. In order to render the two curves comparable 2·1% was deducted from the mean values obtained in the protein-deficient series. 5% of the body weight of water was given making 8·6% the starting-point of the absorption curves. The vertical lines represent the standard error.

In normal rats the absorption of 5·0% of body weight of water by the alimentary canal was practically finished in 60–75 min. This is in agreement with Heller & Smirk's (1932) findings. In protein-deficient rats the absorption of the standard amount of water was terminated in 45–60 min. (Fig. 1).

It is thus quite clear that the rate of water absorption from the gut in hypoproteinaemic rats is not slower than that in normal rats, and cannot therefore be the cause of the delayed onset of the water diuresis in these animals.

Changes in water concentration of plasma and tissues in normal and hypoproteinaemic rats

Changes in the water concentration of plasma and tissues after water administration, in both normal and hypoproteinaemic rats, are shown in Table 1. In normal rats the absorption of water resulted in an early increase of the plasma water content ($t = 2{\cdot}666$, $P < 0{\cdot}02$). This increase reached its maximum at about 45 min. and was accompanied by a fall in the plasma protein concentration (Table 1). In hypoproteinaemic rats no significant changes in the plasma water could be found during the 90 min. of observation, nor were there any significant changes in the plasma protein concentration (Table 1). It would seem that the water left the blood plasma at approximately the same rate as it was absorbed from the alimentary canal.

In normal rats the amount of water in muscle and liver was significantly increased 30 and 45 min. after water administration ($t = 3{\cdot}107$, $P < 0{\cdot}001$; and $t = 3{\cdot}779$, $P < 0{\cdot}001$, respectively). The maximum increase in the water load of these tissues occurred at about 60 min.

In protein-deficient rats (*TT*) no significant changes in the amount of water in the liver could be noticed during the whole period of observation. The increase in the amount of water in muscle was delayed: the first significant increase occurred 60 min. after water administration ($t = 2{\cdot}120$, $P < 0{\cdot}05$) as compared with 30 min. in normal rats.

No significant changes in the amount of water in brain were noted in either *ST* or *TT* rats.

Table 1. *The effect of water administration on the water content of plasma muscle, liver and brain of normal and of hypoproteinaemic rats*

(The values are means and standard errors. Numbers of rats in parentheses.)

Stage of observation	Plasma water content (%)	Muscle water content (%)	Liver water content (%)	Brain water content (%)	Plasma proteins (Kjeldahl) (g./100 ml.)
			Normal rats		
Before water administration	91·4±0·13 (27)	75·8±0·32 (27)	71·4±0·31 (27)	83·6±0·24 (11)	7·06±0·092 (27)
15 min. after water administration	91·9±0·11 (20)	76·9±0·30 (20)	71·5±0·44 (20)	83·0±0·26 (9)	6·66±0·120 (18)
30 min. after water administration	92·3±0·10 (18)	77·0±0·15 (18)	72·0±0·33 (18)	83·9±0·34 (7)	6·54±0·182 (18)
45 min. after water administration	93·0±0·18 (18)	77·4±0·16 (18)	72·5±0·32 (18)	83·7±0·27 (7)	6·48±0·120 (18)
60 min. after water administration	92·5±0·26 (16)	77·8±0·21 (16)	72·3±0·32 (16)	84·3±0·76 (6)	6·49±0·108 (16)
75 min. after water administration	92·5±0·17 (16)	77·4±0·11 (16)	71·7±0·27 (16)	84·0±0·48 (6)	6·48±0·058 (16)
90 min. after water administration	92·6±0·13 (12)	76·8±0·13 (12)	72·2±0·30 (12)	83·9±0·31 (6)	6·48±0·075 (12)
120 min. after water administration	92·0±0·09 (12)	76·3±0·23 (12)	71·8±0·30 (12)	83·4±0·15 (6)	6·64±0·190 (12)
			Hypoproteinaemic rats		
Before water administration	93·2±0·16 (29)	77·2±0·28 (30)	74·9±0·39 (30)	83·3±0·41 (12)	5·12±0·137 (29)
15 min. after water administration	93·5±0·14 (10)	77·5±0·21 (10)	74·8±0·54 (10)	83·8±0·67 (10)	4·66±0·101 (10)
30 min. after water administration	93·5±0·16 (12)	77·1±0·82 (12)	74·6±0·60 (12)	83·5±0·26 (8)	4·75±0·130 (10)
45 min. after water administration	93·7±0·16 (16)	77·6±0·50 (16)	74·1±0·48 (16)	83·5±0·33 (6)	4·73±0·163 (16)
60 min. after water administration	93·7±0·14 (20)	78·4±0·28 (20)	75·0±0·34 (20)	83·4±0·92 (8)	4·86±0·136 (20)
75 min. after water administration	—	—	—	—	—
90 min. after water administration	93·3±0·20 (7)	78·2±0·22 (7)	75·4±0·57 (7)	83·4±0·18 (7)	4·93±0·131 (7)
120 min. after water administration	—	—	—	—	—

Changes in the concentration of electrolytes of plasma and tissues in normal and hypoproteinaemic rats

The concentration of chloride in plasma decreased soon after water administration in normal and hypoproteinaemic rats (Tables 2 and 3). The decrease in the plasma chloride concentration lasted for 30 min. in normal rats, and more than 60 min. in *TT* rats; in other words, it covered in both series the period of time elapsing between that of administration of water and that of the onset of water diuresis. The decrease of chloride concentration in the plasma was not the result of a dilution of the plasma. This is seen from the results obtained on rats fed on *TT* diet (Table 1) where the administration of water by stomach tube did not produce an increase of the plasma water content. Nor can the decrease be explained by an escape of chloride into the muscles or the liver (Tables 2 and 3): in both normal and protein-deficient rats the fall in the plasma chloride concentration was concurrent with a fall in the concentration of chloride in the muscle, while the chloride concentration in the liver remained unaffected. The fall in muscle chloride in the two series of rats lasted as long as that in the plasma. Finally, it will be noted (Tables 2 and 3) that variations in plasma chloride were independent of variations in the concentrations of sodium and potassium in the plasma. These findings agree with those of Priestley (1916), Smirk (1932) and Eggleton (1937) which suggest that water diuresis is preceded by passage of chloride into the gut.

Table 2. *The effect of water administration on the concentration of electrolytes of plasma and tissues in normal rats*

(The values are means and standard errors. Number of rats in parentheses.)

Stage of observation	Cl^- (m-equiv./l. water)	Na (m-equiv./l. water)	K (m-equiv./l. water)	Cl^- (m-equiv./l. water)	Na (m-equiv./l. water)	K (m-equiv./l. water)
		Plasma			Muscle	
Before water administration	83·2±2·34 (27)	128·3±7·05 (18)	5·6±0·29 (17)	20·2±0·47 (27)	29·0±1·48 (17)	156·2±7·73 (17)
15 min. after water administration	72·3±1·63 (20)	112·8±4·04 (10)	4·5±0·31 (9)	17·5±0·67 (20)	25·0±2·27 (9)	155·0±4·57 (9)
30 min. after water administration	69·3±2·04 (18)	112·3±4·56 (12)	5·2±0·41 (7)	18·9±0·99 (18)	26·0±2·73 (11)	145·3±2·87 (7)
45 min. after water administration	82·2±2·80 (18)	134·2±5·32 (15)	6·8±0·66 (13)	22·5±0·92 (18)	36·1±1·69 (13)	134·2±5·62 (13)
60 min. after water administration	82·3±5·54 (16)	137·5±3·90 (12)	5·7±0·44 (12)	22·4±1·23 (16)	36·1±1·94 (12)	147·2±4·41 (12)
75 min. after water administration	86·5±2·16 (16)	137·6±5·57 (16)	5·2±0·26 (16)	23·2±0·66 (16)	37·5±1·98 (16)	158·7±4·60 (16)
90 min. after water administration	78·6±2·62 (12)	128·3±3·09 (12)	6·1±0·40 (12)	20·4±0·74 (12)	32·5±1·12 (12)	154·0±8·01 (12)
120 min. after water administration	84·0±1·87 (12)	131·3±4·00 (12)	5·0±0·26 (12)	20·7±0·75 (12)	29·1±1·55 (12)	157·5±3·67 (12)
		Liver			Brain	
Before water administration	33·6±2·80 (11)	31·0±2·09 (10)	97·8±7·63 (10)	42·7±2·89 (9)	49·0±3·56 (10)	124·0±10·50 (10)
15 min. after water administration	29·0±2·74 (9)	38·1±3·71 (9)	98·0±4·56 (9)	35·3±3·56 (8)	46·1±4·13 (8)	137·8±8·17 (9)
30 min. after water administration	34·0±3·83 (7)	45·5±4·12 (7)	94·9±4·90 (7)	33·4±3·37 (7)	46·1±6·20 (7)	132·7±12·20 (7)
45 min. after water administration	47·0±1·83 (7)	55·6±1·72 (7)	106·0±9·36 (7)	39·0±2·29 (6)	53·0±3·35 (7)	124·0±7·56 (7)
60 min. after water administration	42·0±3·80 (6)	51·4±3·39 (5)	115·5±12·10 (5)	37·7±3·26 (6)	55·3±2·80 (6)	127·5±7·78 (6)
75 min. after water administration	38·4±2·33 (6)	55·4±3·09 (6)	126·1±10·30 (6)	37·3±2·20 (6)	55·5±2·14 (6)	131·6±4·74 (6)
90 min. after water administration	40·5±2·76 (6)	49·2±3·05 (6)	116·6±12·46 (6)	38·0±2·81 (6)	51·4±3·81 (6)	146·5±5·18 (6)
120 min. after water administration	34·8±1·92 (6)	35·0±2·14 (6)	127·0±9·74 (6)	40·3±3·55 (5)	53·7±1·60 (6)	130·9±9·17 (6)

Table 3. *The effect of water administration on the concentration of electrolytes of plasma and tissues in hypoproteinaemic rats*

(The values are means and standard errors. Number of rats in parentheses.)

Stage of observation	Cl^- (m-equiv./l. water)	Na (m-equiv./l. water)	K (m-equiv./l. water)	Cl^- (m-equiv./l. water)	Na (m-equiv./l. water)	K (m-equiv./l. water)
		Plasma			Muscle	
Before water administration	85·9±2·77 (29)	108·3±2·12 (11)	7·1±0·58 (11)	46·7±2·44 (29)	69·5±4·22 (11)	96·6±3·84 (11)
15 min. after water administration	75·6±2·45 (10)	100·0±1·62 (10)	6·8±0·38 (10)	41·7±1·87 (10)	59·5±1·51 (10)	105·5±3·89 (10)
30 min. after water administration	63·4±1·03 (12)	97·1±2·20 (8)	7·1±0·82 (8)	37·6±2·68 (12)	61·5±5·92 (8)	93·7±5·99 (8)
45 min. after water administration	71·9±3·08 (16)	104·2±3·40 (6)	5·8±0·21 (6)	42·1±1·76 (16)	61·4±6·30 (6)	109·0±8·27 (6)
60 min. after water administration	79·2±3·20 (20)	110·8±3·55 (8)	6·4±0·53 (8)	51·7±1·82 (20)	76·8±5·00 (8)	97·4±3·20 (8)
90 min. after water administration	86·8±2·90 (7)	113·6±3·42 (7)	6·4±0·12 (7)	47·9±2·66 (7)	64·7±2·23 (7)	109·5±10·81 (7)
		Liver			Brain	
Before water administration	36·4±2·55 (11)	48·7±3·24 (11)	93·8±3·61 (11)	39·1±3·27 (11)	59·5±3·25 (11)	114·4±4·44 (11)
15 min. after water administration	39·5±1·11 (10)	52·3±2·64 (10)	91·8±6·22 (10)	39·5±1·87 (10)	60·9±2·02 (10)	118·5±2·90 (10)
30 min. after water administration	34·6±3·04 (6)	49·1±3·40 (6)	89·7±5·35 (6)	35·7±2·77 (8)	59·1±4·83 (8)	118·8±10·56 (8)
45 min. after water administration	38·6±4·96 (6)	46·6±2·70 (6)	92·5±2·48 (6)	35·1±1·34 (6)	58·6±1·96 (6)	110·5±6·82 (6)
60 min. after water administration	39·3±3·61 (8)	58·5±5·77 (8)	98·5±7·76 (8)	39·5±2·99 (8)	60·9±5·38 (8)	112·6±6·23 (8)
90 min. after water administration	39·0±0·97 (7)	56·1±1·43 (7)	102·2±13·11 (7)	40·0±1·67 (7)	62·8±3·24 (7)	116·0±5·60 (7)

The plasma chloride concentrations were normal again in normal rats 45 min. after water administration, and in hypoproteinaemic rats 60 min. after water administration, and then remained at this level for the remaining period of observation. Chloride concentration in muscle increased over the mean values of control rats, 45 min. after water administration in normal rats ($t=3·010$, $P<0·01$) and 60 min. after water administration in hypoproteinaemic rats ($t=4·031$, $P<0·001$). The increase in the chloride concentration of muscle in the two series corresponded to the onset of water diuresis.

It is thus clear that in normal as well as in hypoproteinaemic rats a water diuresis can be divided into two stages: (*a*) a prediuretic or absorptive period, during which there was a fall of the chloride concentration in both plasma and muscle, independent of changes in their hydration; (*b*) an excretory period with increased water load in plasma and tissues during which the chloride concentration returned, nevertheless, to normal values in plasma and reached higher than normal values in the muscle.

Changes in the extracellular-fluid phase of tissues

The changes in the water content of plasma and the tissues, associated with changes in the concentration of electrolytes, led to variations in the amount of extracellular fluid of tissues.

It would seem from Tables 4 and 5 that the partition of water between the extracellular- and intracellular-fluid phase during a water diuresis differs in normal and hypoproteinaemic animals.

In normal rats three stages could be recognized (Fig. 2): an initial decrease of the extracellular-fluid phase of muscle (up to 30 min. after water administration) followed by a significant increase (45–75 min.) ending in a slow return to normal values (90–120 min.). When the changes of the extracellular-fluid phase are correlated with the rate of renal water excretion, it can be seen that the initial decrease of extracellular-fluid phase corresponded to the prediuretic period and the increase to the peak of the diuresis.

In the hypoproteinaemic rats, although the average changes in extracellular-fluid phase of

Table 4. *The effect of water administration on the extracellular- and intracellular-fluid phases (chloride and sodium) of tissues in normal rats*

(The values are means and standard errors. Number of rats in parentheses.)

Stage of observation	Extracellular-fluid phase (ml./100 g.)		Intracellular-fluid phase (ml./100 g.)	
	Cl^-	Na	Cl^-	Na
		Muscle		
Before water administration	16·8±0·50 (27)	16·9±0·53 (17)	59·0±0·50 (27)	58·8±1·11 (17)
15 min. after water administration	15·3±0·27 (20)	15·4±0·47 (9)	61·5±0·40 (20)	61·5±1·23 (9)
30 min. after water administration	18·9±0·90 (18)	18·0±1·00 (11)	58·2±0·85 (18)	59·0±1·04 (11)
45 min. after water administration	20·8±0·85 (18)	21·1±0·86 (13)	56·5±0·89 (18)	56·3±0·88 (13)
60 min. after water administration	20·5±0·88 (16)	20·5±1·18 (12)	57·3±0·93 (16)	57·3±1·13 (12)
75 min. after water administration	20·6±0·66 (16)	20·7±0·70 (16)	56·7±0·63 (16)	56·7±0·64 (16)
90 min. after water administration	18·3±0·80 (12)	20·4±0·78 (12)	58·5±0·78 (12)	56·5±0·82 (12)
120 min. after water administration	17·1±0·49 (12)	16·5±0·74 (12)	59·3±0·59 (12)	59·7±0·80 (12)
		Liver		
Before water administration	22·6±2·50 (11)	21·3±1·55 (10)	48·8±2·05 (11)	50·1±2·03 (10)
15 min. after water administration	25·9±2·76 (9)	23·8±2·42 (9)	45·6±1·56 (9)	47·6±1·20 (9)
30 min. after water administration	34·4±3·60 (7)	32·6±3·57 (7)	37·6±3·65 (7)	40·6±3·63 (7)
45 min. after water administration	32·0±1·40 (7)	33·4±3·90 (7)	40·8±1·43 (7)	39·2±3·80 (7)
60 min. after water administration	27·6±2·91 (6)	26·3±4·32 (5)	44·7±3·40 (6)	46·0±4·93 (5)
75 min. after water administration	30·1±2·39 (6)	30·2±3·15 (6)	41·6±1·76 (6)	41·3±3·03 (6)
90 min. after water administration	30·6±1·87 (6)	32·9±2·50 (6)	41·6±3·40 (6)	39·4±2·53 (6)
120 min. after water administration	31·5±1·70 (6)	28·1±1·95 (6)	40·3±1·75 (6)	43·7±2·30 (6)

Table 5. *The effect of water administration on the extracellular- and intracellular-fluid phases (chloride and sodium) of tissues in hypoproteinaemic rats*

(The values are means and standard errors. Number of rats in parentheses.)

Stage of observation	Extracellular-fluid phase (ml./100 g.)		Intracellular-fluid phase (ml./100 g.)	
	Cl^-	Na	Cl^-	Na
		Muscle		
Before water administration	44·9±1·90 (29)	43·5±2·08 (11)	32·3±1·64 (29)	33·8±0·70 (11)
15 min. after water administration	41·9±2·16 (10)	38·8±1·24 (10)	35·5±1·86 (10)	38·7±1·24 (10)
30 min. after water administration	44·2±3·41 (12)	42·0±3·90 (8)	32·7±3·00 (12)	35·0±3·72 (8)
45 min. after water administration	46·2±3·51 (16)	44·0±4·66 (6)	31·4±3·40 (16)	33·5±4·12 (6)
60 min. after water administration	49·1±2·42 (20)	51·5±4·67 (8)	30·0±2·40 (20)	26·9±3·67 (8)
75 min. after water administration	—	—	—	—
90 min. after water administration	41·1±2·45 (7)	42·2±2·29 (7)	37·0±2·72 (7)	36·0±2·66 (7)
120 min. after water administration	—	—	—	—
		Liver		
Before water administration	35·4±2·66 (11)	32·6±1·91 (11)	39·5±2·52 (11)	42·2±2·14 (11)
15 min. after water administration	39·8±1·22 (10)	37·9±2·75 (10)	35·0±1·15 (10)	37·0±3·03 (10)
30 min. after water administration	40·8±3·14 (6)	33·4±2·38 (6)	33·8±3·12 (6)	41·2±2·62 (6)
45 min. after water administration	39·4±3·42 (6)	32·3±2·02 (6)	34·9±3·59 (6)	41·8±1·87 (6)
60 min. after water administration	40·4±3·66 (8)	37·2±3·76 (8)	34·8±3·54 (8)	38·0±3·67 (8)
75 min. after water administration	—	—	—	—
90 min. after water administration	33·6±1·07 (7)	36·0±1·64 (7)	41·8±0·83 (7)	39·4±1·37 (7)
120 min. after water administration	—	—	—	—

muscle resembled closely, both in magnitude and direction, those observed in the normal animals, such changes cannot be regarded as significant owing to the large standard error of each group (Table 5).

Changes in the intracellular-water phase of tissues

It has been shown in the present series of experiments on normal rats that during a water diuresis the total water load of muscle and its extracellular-fluid phase increased in a parallel manner. But it is not clear whether these simultaneous increases are of the same magnitude, i.e. whether the increase of the extracellular-fluid phase can be accounted for by the increase of the extra water load of the muscle.

Comparing the mean values for extracellular fluid and the total muscle water load (Tables 1 and 4) it will be seen that, whereas the extracellular-fluid phase increased from 16·8 to 20·5 ml./100 g. fat-free muscle (i.e. by 3·7 ml.) in 60 min., that of total water content increased from 75·8 to 77·8 ml./100 g. fat-free muscle (i.e. by 2·0 ml.) in the same time. The differences in the increments represents the changes in the amount of intracellular-fluid phase (Table 4).

It is thus evident that during the second or excretory stage of water diuresis an increase in the extracellular-fluid phase was associated with a decrease in the amount of intracellular fluid with a concurrent increase in the extra water load of the muscle (Fig. 2). In agreement with this finding is the observation that the loss of intracellular water was accompanied by a loss of potassium (Table 2). This finding agrees with that of Gamble, Blackfan & Hamilton (1925), who showed that water diuresis may cause a marked urinary excretion of endogenous potassium.

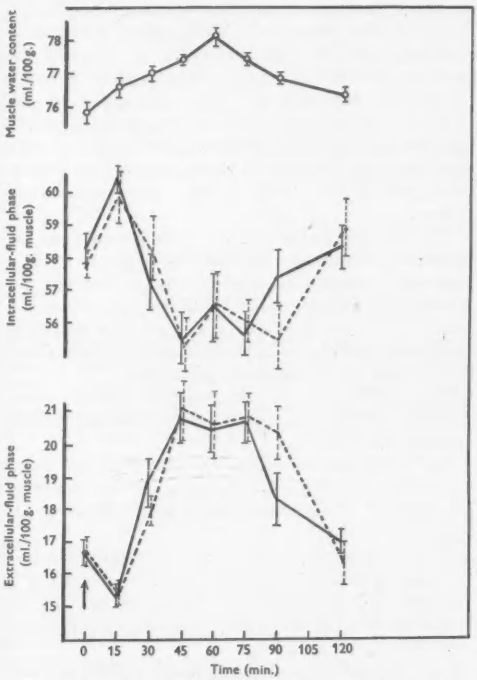

Fig. 2. Changes of total water content and of the extracellular- and intracellular-fluid phases in the muscle of normal rats. At ↑ 5% of body weight of water was administered. •—•, extracellular- and intracellular-fluid phases estimated in terms of chloride space; •- - - -•, extracellular- and intracellular-fluid phases estimated in terms of sodium space; ○—○, total muscle water content. The vertical lines represent the standard error.

In hypoproteinaemic animals, on the other hand, no clear changes in the intracellular-fluid phase of the tissues could be shown. The partition of water between the extracellular- and the intracellular-fluid phases in the tissues during a water diuresis was thus completely different in normal and hypoproteinaemic rats.

DISCUSSION

It would appear that in normal rats well-defined changes in the partition of water between the extracellular- and the intracellular-fluid phase of skeletal muscles corresponded to certain stages in the process of water diuresis. Following the administration of water by stomach tube, and preceding the onset of diuresis, there was a significant decrease in the extracellular-fluid phase of the muscle. When the absorptive period was reaching completion and when the renal excretion of the administered water had started, the well-known increase of the muscle water load was observed. A significant increase in the extracellular-fluid phase concurrent with a decrease of the intracellular-fluid phase could be demonstrated at this stage. When the excretion of the extra water was nearly completed there was a return of the muscle water load and of its extracellular- and intracellular-fluid phases to normal values.

These results compare with Eichelberger's (1941) findings on unanaesthetized dogs, but are in apparent contrast to those of Eggleton (1937) on anaesthetized cats. Eichelberger (1941) injected 0·9% NaCl solution intravenously into dogs, and observed that during the period of full diuresis there was a significant increase in the extracellular-fluid phase of the muscle, and a slight decrease in its intracellular-fluid phase. In other words, the partition of water in Eichelberger's (1941) dogs corresponded to that observed during the excretory stage in the present series of normal rats. Eggleton (1937), on the other hand, found that the injection of 6% of the body weight of water 'into a loop of small intestine brought to the surface through a small abdominal opening' produced an increase in the total muscle water content accompanied by a decrease of its extracellular-fluid phase. It should be pointed out, however, that none of Eggleton's (1937) cats was excreting the administered water; in fact, some of them had their ureters tied.

The difference between Eggleton's (1937) findings on the one hand, and those of Eichelberger (1941) and of the present series of investigations on the other, thus lies clearly in the fact that in Eggleton's (1937) cats water excretion was prevented. It is therefore likely that Eggleton's (1937) findings apply to the prediuretic or reabsorptive period only, a stage characterized by a dilution of the plasma solids and by a fall of the chloride concentration in both plasma and muscles, resulting in a decrease of the extracellular space.

In hypoproteinaemic rats the administration of the standard amount of water did not produce a significant increase of the water level in the plasma, in spite of the fact that water had been absorbed at the same rate at least as by normal rats. During the absorptive period there was a marked fall of the

plasma and muscle chloride concentrations, but in contrast to the normal animals there was no significant decrease in the extracellular-fluid phase of the skeletal muscle. Nor was there any significant increase of the extracellular-fluid phase of muscle during the post-absorptive or excretory period. It would thus seem that the tissue oedema that existed initially in the hypoproteinaemic animals could not be increased to any significant extent by further administration of water.

It has indeed been suggested that in muscles in which the concentrations of chloride and sodium are increased and that of potassium decreased (i.e. in which an increase of the extracellular-fluid phase at the expense of the intracellular-fluid phase obtains) the extracellular-fluid phase should not be identified with an ultrafiltrate of plasma (Manery, Danielson & Hastings, 1938) but with connective tissue diluted with serum filtrate. The extracellular-fluid phase of connective tissue has been estimated to amount to almost 100% (Manery & Hastings, 1939). This interpretation may explain why an increased water load did not produce any significant increase in the extracellular-fluid phase of the muscle.

This interpretation would also provide an explanation for the delayed and diminished water diuresis displayed by hypoproteinaemic rats.

In normal rats the administration of a standard amount of water resulted in a dilution of the plasma, which according to accepted theories is indirectly responsible for the reduction of the rate of tubular water reabsorption. There is a time lag between the height of gastro-intestinal water absorption and the height of the diuresis, i.e. a period during which the water absorbed is lodged in the extracellular-fluid phase of tissues, from where it ultimately returns to the blood stream to be excreted by the kidneys. In hypoproteinaemic rats the administration of a standard amount of water did not result in a further dilution of the plasma: the rate of tubular water reabsorption was thus not affected. This would explain why the water diuresis was delayed in its onset, and why the diuresis was partly regulated by an enhanced glomerular filtration rate (Dicker *et al.* 1946). As the rate at which water was absorbed from the gut was not slower than in normal rats, and as the extracellular-fluid phase of skeletal muscle or liver seemed to be unable to store the amount of water absorbed, it must have been stored elsewhere. Few of the rats in this series showed any free fluid at a post-mortem examination, but nearly all of them displayed perirenal and retroperitoneal connective tissues so much distended with fluid as to give them a gelatinous consistency. In several cases mediastinal connective tissue had the same appearance. It must, therefore, be assumed that in hypoproteinaemic rats water administered by stomach tube is not held in the extracellular-fluid phase of muscle or liver, but that it collects in the deep connective tissue.

SUMMARY

1. A standard amount of water (5% of their body weight) was administered by stomach tube to normal and hypoproteinaemic rats, and the partition of the absorbed water between the plasma and the extracellular- and intracellular-fluid phases of certain tissues (muscle, liver and brain) was investigated.

2. In normal rats the administration of water produced effects which led to the division of the process of water diuresis into three distinct periods:

(*a*) The absorptive period, preceding the onset of diuresis and characterized by a dilution of the plasma accompanied by a fall in the chloride and the sodium concentration of both plasma and tissues, resulting in a decrease of the extracellular-fluid phase.

(*b*) The excretory period, corresponding to the height of the water diuresis accompanied by a marked increase in the chloride and sodium concentration of muscle and a decrease of potassium, resulting in an increase of the extracellular-fluid phase and a decrease in the intracellular-fluid phase of the tissue, i.e. oedema of the tissue.

(*c*) The terminal period: return to normal values for chloride, sodium and potassium concentration in plasma and tissues.

3. In hypoproteinaemic rats water administered was absorbed at least at the same rate as in normal animals. The water absorption was finished in less than 60 min.

4. In hypoproteinaemic rats the administration of water produced a diuresis which was delayed in its onset and diminished in its volume. This abnormal response to water administration could be explained by the following findings:

(*a*) The administration of water did not produce any further increase in the plasma water content.

(*b*) No significant changes in the amount of extracellular-fluid phase per 100 g. fat-free muscle could be found.

(*c*) The water absorbed was 'visibly' collected in the perirenal and retroperitoneal connective tissue, which was so much distended as to have a gelatinous consistency.

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Chemical Constitution and Insecticidal Action

1. ORGANIC SULPHUR COMPOUNDS

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Until the discovery of the newer synthetic insecticides of the chlorinated hydrocarbon class, such as DDT and γ-benzene hexachloride, organic compounds of sulphur had attracted considerable attention, and this work led to the commercial introduction of the long-chain alkyl thiocyanates, to phenothiazine (originally as an insecticide but later developed as an anthelmintic) and more recently to tetraethylthiuram sulphide, an acaricide used in the treatment of scabies and mange. During the last ten years we have made an extensive investigation of the insecticidal action of organic sulphur compounds, in collaboration with the Hawthorndale Laboratory of Jealott's Hill Research Station (Imperial Chemical Industries Ltd.) where the entomological testing was carried out. Although these investigations did not result in the introduction of any new commercial insecticides, nevertheless, marked insecticidal activity was found in certain types, and the correlation of structure with activity presented points of considerable interest. It is the purpose of this communication to record some of our observations on this subject.

METHODS

All compounds were submitted to routine sorting tests, and from the results the more promising were selected for extended evaluation. The extended tests necessitated the preparation of a variety of types of emulsions and dispersions (Collie, Ellingworth & Robertson, 1939; Collie, Davies & Sexton, 1939; Harland & Sexton, 1943) suitable for the practical application of the poisons. This aspect of the research is mentioned only so far as it provides data confirming the higher activities found by the sorting tests and illustrates the selective poisoning effect on insect species.

Contact insecticides. The sorting tests were carried out in the Tattersfield spraying apparatus using aphids (*Macrosiphum* spp. except where otherwise stated, the choice of species being governed by its availability at the time of the test), or adult blowflies (*Calliphora erythrocephala* Meig.) as the test insects. The compound to be tested was dissolved in ethanol, and poured into a 0·1% aqueous solution of a surface-active agent of the sulphonated hydrocarbon type, the concentrations being such that the final spray contained the substance dispersed in 50% ethanol. In certain instances it was necessary to use acetone instead of ethanol. As it was appreciated that the organic solvent might play a significant part in the apparent toxicities, controls were carried out with sprays of similar composition from which the poison had been omitted; the 'absolute mortality' due to the substance under test was calculated by the formula:

$$\text{Percentage absolute mortality} = \frac{(\text{number killed by poison spray}) - (\text{number killed by control spray})}{(\text{total number of insects}) - (\text{number killed by control spray})} \times 100.$$

In spite of this adjustment, the ethanol may have played a part in altering penetration of the insect cuticle by the poison (Hurst, 1943) so that the figures of 'absolute mortality' obtained may have been, considered as absolute values, false. It is felt, however, that for closely related

Table 1. *Insecticidal activities of 2-alkylthiobenzthiazoles*

Alkyl group (*R*, formula I)	Percentage absolute mortality: Blowflies at 0·5%	Aphids (*Macrosiphum* spp.) at 0·2%	*Sitophilus granarius* (biscuit method)
H	13*	—	0
CH_3	100	86	99·4
C_2H_5	100*	21	100
C_3H_7†	100	30	23·3
C_4H_9	96	17	2·7
C_5H_{13}	41	3	0
C_7H_{15}	37	0	7
C_8H_{17}	—	9	0
C_8H_{17} (*sec.*)	—	17‡	0
$C_{12}H_{25}$	4	45	0
$C_{16}H_{33}$	0*	17	0
$(CH_3)_2CH$	100*	24	50
$(CH_3)_2CHCH_2$	92*	86	7
C_5H_{11} (*iso*)	—	17	—
$CH_2{:}CHCH_2$	99*	52	13·6
$C_6H_5CH_2$	62*	6	10·5
HOC_2H_4	18*	—	83§
$C_2H_5OC_2H_4$	86*	30‡	—
Standards: Rotenone	100*	100	—
Lead arsenate	—	—	58·1
$C_{12}H_{25}SCN$	100	100	—

* Sprays contained 25% acetone instead of 50% ethanol.
† C_3H_7, C_4H_9, C_5H_{11}, etc., indicate primary straight-chain alkyl radicals unless otherwise stated.
‡ Against *Doralis rumicis* L. (=*Aphis rumicis* L. (*fabae* Scop.)) in place of *Macrosiphum* spp.
§ Lead arsenate in this experiment gave 88·7% absolute mortality.

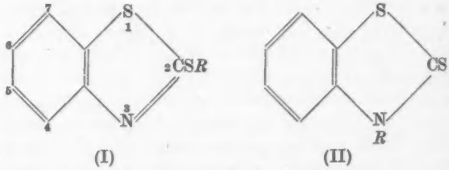
groups of compounds such as those examined the 'absolute mortality' figures give a reasonably adequate comparison of the toxicities of individual members of such groups. That the toxicities were genuine was later shown by the examination of special preparations of the more active compounds.

Stomach poisons. The sorting tests were carried out against *Sitophilus granarius* L. (grain weevils) by a technique known as the 'biscuit' method. One part of the poison was mixed with 50 parts of flour and the whole made into a dough with 30 parts of water; the dough was dried in thin layers and broken up to provide 'biscuits' on which the insects were allowed to feed. Lead arsenate, similarly compounded, was used as a standard of comparison.

RESULTS

Tests on S-alkylated derivatives of 2-thiolbenzthiazole and 2-thiolquinoline

The contact insecticidal activity of alkyl thiocyanates (Bousquet, Salzberg & Dietz, 1935) prompted the exploration of other compounds containing the sequence of atoms —S—C—N—, and derivatives of types (I) (Collie, Ellingworth & Robertson, 1939) and (II) (*R*=alkyl) were therefore investigated. Preparative methods for type (II) have been described elsewhere (Sexton, 1939; Reed, Robertson & Sexton, 1939). (The numbering of the benzthiazole ring is indicated in formula (I).) The results of the preliminary tests with the *S*-alkylated derivatives (I) are given in Tables 1 and 2. Against blowflies, toxicity is at a maximum at C_3 in the normal series: for aphids, the methyl and *iso*butyl compounds were the most toxic, the normal C_5 to C_8 compounds being practically inactive. There was,



(I) (II)

however, appreciable activity with the *iso*octyl and dodecyl derivatives, but the hexadecyl was again inactive. For the grain weevil there was a marked fall in toxicity after the methyl, ethyl and β-hydroxyethyl compounds.

Table 2. *Effect of concentration on toxicity to blowflies of aqueous acetone dispersions of homologous 2-alkylthiobenzthiazoles*

Alkyl group (*R*, formula I)	Percentage absolute mortality: Concentration (%) 0·5	0·3	0·15	0·075	0·05
CH_3	100	98	34	10	—
C_2H_5	100	94	49	16	—
C_3H_7	100	99	85	10	—
$(CH_3)_2CH$	—	—	18	—	0
$(CH_3)_2CHCH_2$	—	—	13	—	0
$CH_2{:}CHCH_2$	—	—	40	—	23
Rotenone	—	—	—	—	100

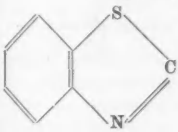
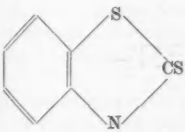
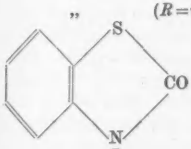
2-Ethylthiobenzthiazole, because of its toxicity to blowflies, was subjected to further evaluation. Its insecticidal effect was confirmed by the following findings in separate experiments: (*a*) a 40 % solution in odourless kerosene gave a 68 % kill when sprayed on houseflies (*Musca domestica* L.), (*b*) emulsified preparations gave kills of 64–98 % at the same strength, (*c*) a 2 % solution in kerosene gave a 81 % kill of mosquito larvae, and (*d*) there was moderate toxicity to bedbugs (*Cimex lectularius* L.).

The effect of substitution in the benzene ring was examined in the cases of the 2-alkylthio derivatives (C_1 to C_4) of 6-amino-, 6-nitro- and 5-chloro-benzthiazoles. The results will not be given in detail, but the general effect was a reduction in insecticidal activity, which was least marked in the case of the chloro compounds, against blowflies.

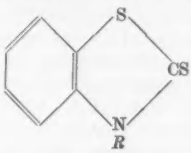
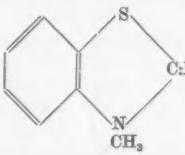
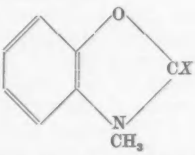
Tests with S-alkylated derivatives of 2-thiolquinoline and of 2-thiolbenzoxazole

The chemical similarity between thiazole and the corresponding pyridine derivatives suggested that this might also extend to their biological action. Accordingly several quinoline derivatives, analogous to the benzthiazole derivatives already examined, were tested against the standard insects. The results, which are not given in detail, showed that the general order of toxicity was lower than for the isosteric thiazole compounds. A notable feature was that, whereas alkylation of the thiol group in the 2-thiolbenzthiazole series raised the toxicity to the grain weevil, a reduction occurred with the quinoline compounds. The results with the other isosteric series, the benzoxazoles, showed, among the limited number

Table 3. *Insecticidal activity of alkylated benzthioazole derivatives*

Compound	Percentage absolute mortality						
	Blowflies		*Doralis rumicis*		*Doralis pomi*		*Sitophilus granarius**
	(0·5 %)	(0·2 %)	(0·2 %)	(0·1 %)	(0·2 %)	(0·1 %)	
 C.SCH$_3$	100	73	11	8	32	—	99·4 (58·1)
 CS (R=CH$_3$)	0	13	73	55	100	73	11·5 (82·3)
,, (R=C$_2$H$_5$)	12	—	70	—	—	—	27·1 (82·3)
,, (R=C$_3$H$_7$)	7	—	0	—	—	—	16·7 (67·7)
,, (R=C$_5$H$_{11}$)	0	—	10	—	—	—	8·3 (67·7)
 CO (R=CH$_3$)	25	—	20	—	—	—	90·5 (82·3)
,, (R=C$_2$H$_5$)	12	—	70	—	—	—	—

The following had very low toxicities both as contact insecticides and stomach poisons:

 CS (R=allyl, benzyl),  C:NH,  CX (X=O, S)

* The figures in brackets in this column give the percentage absolute mortality with a lead arsenate bait.

of compounds examined, results parallel to those obtained with the benzthiazole derivatives in that blowflies and grain weevils were more susceptible than aphids.

Tests with N-alkylated derivatives of 2-thiolbenzthiazole

The tests in this series were confined to the lower *N*-alkylated derivatives of type (II) which were readily obtainable by methods already mentioned. An important difference in the relative susceptibility of different insects to the isomeric *S*- and *N*-alkylated pairs was revealed, for whereas the *S*-alkyl derivatives had low toxicity to aphids and high toxicity to blowflies, the *N*-methyl compound, which received the most study, was highly toxic to aphids and relatively non-toxic to blowflies. The results with these and related compounds are summarized in Table 3. In the *N*-alkyl series, the lowest member was the most active aphicide. It will be seen from Table 3 that replacement or interchange of >S, >O and >NH groups could exert marked effects, and the S derivative was not always the most toxic.

The aphicidal properties of the *N*-methyl compound, 2-thio-3-methylbenzthiazolone, were investigated thoroughly, and clear evidence was obtained that the activity was not due to the ethanol in the spray; indeed this may even have lowered the aphicidal activity. A concentrated emulsion was prepared by dissolving 10 parts of 2-thio-3-methylbenzthiazolone in 55 parts of cottonseed oil and emulsifying with 25 parts of water with the aid of 10 parts of a sulphonated cod oil. This emulsion, diluted with 0·1 % 'Agral' Liquid Wetter, was sprayed on plants infested with several species of aphids. High kills of the insects were obtained, sometimes even at spray concentrations of less than 0·025 % active ingredient. Other emulsions gave similar results. The aphicidal activity was, however, generally lower than that of dodecyl thiocyanate, and the practical utilization of this compound was limited by occasional damage to plants.

Tests on carbamates and thiocarbamates

An obvious extension of the types of compound containing adjacent S, C and N atoms was to derivatives of dithiocarbamic acid. Since, moreover, interchange of sulphur and oxygen in this class had not led to marked alterations in fungistatic activity (Davies & Sexton, 1946), or even to a more limited extent in selective herbicidal activity (Templeman & Sexton, 1946), a comparison of the insecticidal activity of carbamates and thiocarbamates was necessary. The results of contact tests are given in Table 4. As stomach poisons against *Sitophilus granarius* nearly all were less toxic than lead arsenate.

Table 4. *Contact insecticidal activity of derivatives of carbamic acid*

Compound	Percentage absolute mortality: Blowflies (0·5 %)	Aphids (0·2 %)
$C_6H_5NH.COOCH_3$	58	4 (*a*)
$C_6H_5NH.COOC_2H_5$	87	0 (*a*)
$C_6H_5NH.CSOCH_3$	96	23 (*a*)
$C_6H_5NH.COSCH_3$	81	0 (*a*)
$C_6H_5NH.COSC_2H_5$	100	4 (*a*)
$C_6H_5NH.CSOC_3H_7$	92	86 (*b*)
$C_6H_5NH.CSOC_4H_9$	55	11 (*b*)
$C_6H_5NH.CSSCH_3$	31	0 (*a*)
$C_6H_5NH.CSSC_2H_5$	76	28 (*a*)
$C_6H_5NH.CSSC_4H_9$	84	45 (*a*)
$C_6H_5NH.CSSCH_2C_6H_5$	57	0 (*a*)
$C_6H_5(CH_3)N.COOCH_3$	2	0 (*b*)
$C_6H_5(CH_3)N.COOC_2H_5$	40	6 (*b*)
$C_6H_5(CH_3)N.CSSCH_3$	9	17 (*b*)
$C_6H_5(CH_3)N.CSSC_2H_5$	0	56 (*b*)
$C_6H_5(C_2H_5)N.COOCH_3$	16	23 (*b*)
$C_6H_5(C_2H_5)N.COOC_2H_5$	59	0 (*b*)
$C_6H_5(C_2H_5)N.CSSCH_3$	28	27 (*b*)
$C_6H_5(C_2H_5)N.CSSC_2H_5$	6	24 (*b*)
$(CH_3)_2N.COOCH_3$	0	6 (*b*)
$(CH_3)_2N.COOC_2H_5$	0	3 (*b*)
$(CH_3)_2N.CSSCH_3$	—	3 (*b*)

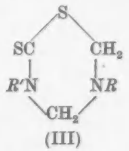
(*a*) *Macrosiphum* spp. (*b*) *Doralis rumicis* L.

Tests on thiocarbimide (isothiocyanate) *derivatives*

The insecticidal activity of thiocyanates differs from that of the corresponding thiocarbimides (*iso*-thiocyanates), just as does the fungistatic activity. Though dodecyl thiocyanate is a powerful contact insecticide, the corresponding *iso*thiocyanate is much less active: experiments with bedbugs and houseflies have shown that it is almost without action on these insects.

In the aromatic series, the thiocarbimides are powerful insecticides (Goldschmidt & Martin, 1937), and the α-naphthyl compound has been developed commercially as a fly spray. The starting point for the results recorded here was phenylthiocarbimide. During a search for new compounds having a high toxicity to locusts, it was observed that this substance was highly toxic when used in a poison bait, but its volatile nature precluded its practical application. Attention was, therefore, directed to less volatile derivatives which might be expected to break down to phenylthiocarbimide *in vivo*, and the reaction products of phenyldithiocarbamic acid with formaldehyde and ammonia or primary amines, the so-called 'carbothialdines', were studied in some detail. During the course of this work their constitution as reduced thiadiazines (III) was established (Ainley, Davies, Gudgeon, Harland & Sexton, 1944). The first substance examined was 'phenylmethylene carbothialdine' ($R = H$, $R' = C_6H_5$), which was obtained as an amorphous powder by treating ammonium phenyldithiocarbamate with formaldehyde under controlled conditions. The crude pro-

duct, which could not be purified because of its instability, proved highly toxic to locusts. More stable, crystalline compounds of the same type were obtained by using primary amines in place of ammonia, thus giving 'carbothialdines' in which R=alkyl: the preparation and properties of these compounds are described elsewhere (Ainley *et al.* 1944; Gudgeon, Harland & Sexton, 1943). In testing as locust poisons, the substances were ground with



(III)

an equal weight of talc, and the resulting 50 % dusts were made into baits by mixing with bran, molasses and water in the proportions 1 : 56 : 2 : 84. Locusts (*Locusta migratoria migratorioides* R & F. 3rd instar hoppers) were allowed to feed on the mixtures, and the mean mortality determined after 20 hr.: a similarly compounded sodium arsenite bait was used as a standard. The results with a series of compounds (Table 5) showed that substitution in the phenyl

Table 5. *Toxicity of compounds of formula (III) to locusts*

Constitution		Mean percentage mortality after 20 hr. (sodium arsenite = 8 %)
R'	R	
C_6H_5	CH_3	100
	C_2H_5	7
	C_3H_7	100
	C_4H_9	53
	$(CH_3)_2CHCH_2$	68
	*cyclo*hexyl	<10
	C_2H_4OH	100
	$—CH_2CH_2—$*	98
	$—(CH_2)_6—$*	<10
$(p)CH_3C_6H_4$	CH_3	88
$(p)ClC_6H_4$	CH_3	62
$(p)HOC_6H_4$	CH_3	<10
CH_3	CH_3	88
	C_2H_5	74
	$—CH_2CH_2—$*	<10
C_2H_5	C_2H_5	74

* These are double molecules comprising two heterocyclic nuclei united by the divalent group R.

group reduced the toxicity, and that the nature of the group R also had a marked effect. The result with the second compound in Table 5 appears anomalous. Further tests, in which the amount of poison in the bait was reduced showed that no compound was more toxic than (III) ($R=CH_3$, $R'=C_6H_5$). This was next evaluated against different insects, when it was observed to be highly toxic to crickets and to the large white cabbage butterfly caterpillar (*Pieris brassicae* L.). It was of low toxicity to winter moth larvae (*Operophtera brumata* L.), and non-toxic to the diamond back moth (*Plutella maculipennis* Curt.) and to aphids. This compound, therefore, provides another outstanding example of selective insecticidal action.

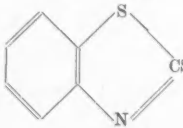
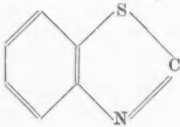
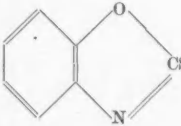
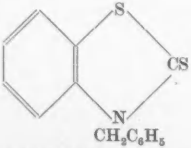
The relative susceptibility of different insect species

The previous tables show that individual compounds have considerable selective insecticidal action against aphids or blowflies. This is characteristic of organic compounds, not only when employed as contact insecticides, but also, as can be seen from the rapidly growing literature on the subject, in their antibacterial, antiprotozoal, fungicidal and herbicidal activities. It is further illustrated in the case of insecticidal action by the figures in Table 6 where stomach poison tests are considered. The first three columns refer to tests by the 'biscuit' method, and the last five columns to tests in which various insect larvae were allowed to feed on foliage sprayed with dispersions of the insecticides. In these tests the mortality by lead arsenate, which was used as a standard of comparison, was 100 % unless otherwise indicated by a figure in brackets. An outstanding example of this selective toxicity is the first compound in the list, 2-methylthiobenzthiazole, which was very toxic to *Sitophilus granarius*, *Ephestia kühniella*, vanessids and *Hypocrita jacobaeae*, but relatively non-toxic to *Corcyra cephalonica* and *Operophtera brumata*.

DISCUSSION

Läuger, Martin & Müller (1944) have put forward the hypothesis regarding contact insecticidal action that the molecule should contain a grouping responsible for its toxic action, and a 'carrier' grouping conferring lipid solubility enabling the compound to pass through the fatty and lipoprotein layers of the insect cuticle. It is of interest to examine this hypothesis further, particularly in the light of the results now recorded. As Läuger *et al.* (1944) point out it is difficult to decide in complicated molecules such, for example, as the pyrethrins, which part represents the toxic grouping and which the lipophilic carrier. We would prefer to speak of (*a*) the chemically functioning group or groups of the molecule, and (*b*) the physico-chemical properties, for example, of lipid and water solubility, surface activity, etc. of the molecule as a whole. By chemically functioning group is meant the group which is responsible for attachment to cell constituents. The force of attachment may not be strong in absolute terms, but if the union is of sufficient stability to disturb a delicate balance of dynamic biochemical processes then biological activity may result. This conception of the dual contribution towards biological activity should not be applied too rigidly for the two factors may, to

Table 6. *Percentage absolute mortality (stomach poison tests) by several compounds against different insect larvae*

Compound	Tests by 'biscuit' method			Tests on foliage				
	Flour moth (*Ephestia kühniella* Zell.)	Rice moth (*Corcyra cephalonica* Staint.)	Grain weevil (*Sitophilus granarius* L.)	Winter moth (*Operophtera brumata* L.)	Small tortoise-shell (*Aglais urticae* L.)	Peacock (*Nympha-lisio* L.)	Cinnabar (*Hypocrita jacobaeae* L.)	Diamond back moth (*Plutella maculipennis* Curt.)
	100 (25·1)*	26·5 (88·7)	99·4 (58·1)	13·8	100	97·5	100	—
	81 (25·1)	—	100 (58·1)	19·5	100	91	74·7	—
	23·1 (20·6)	—	83 (88·7)	—	—	0	0	7·1 (27·1)
	—	—	95·2 (13)	—	—	0	—	—
	—	—	0·7 (66·6)	—	—	—	—	9·3 (27·1)
$C_6H_5NHCSSC_4H_9$	—	—	—	44·5	—	2·2	—	—

* Mortality with lead arsenate (see text).

some extent, be interdependent. The chemically functioning groups may have their contribution to make towards physical properties, particularly when the lesser interatomic forces such as those of the hydrogen bond are concerned. Physical causes may predominate in insecticidal action, and in these instances certain characteristic effects can be observed. These have been considered in relation to biological activity generally on a thermodynamic basis by Ferguson (1939), and they include the effects on variation in homologous series, including the peak effect noted in the aphicidal properties of alkyl thiocyanates, and in several instances reported in this paper. Another characteristic manifestation of physical mechanisms is the varying response of different biological species (cf. Ferguson, 1939) to the same compound. Marked specificity of insecticidal action is recorded in this paper. This is probably a more general effect of organic insecticides than has been realized, for the more potent contact insecticides are usually used at such high strengths relative to their absolute potency that selective effects may be obscured. The chemically functioning groups in the compounds reported here are not identical, although the compounds nearly all contain the sequence of atoms —S—C—N—. In the powerfully insecticidal aliphatic thiocyanates, the critical feature of the molecule is probably the ability of the carbon atom of the —SCN group to accept electrons (Hoggarth & Sexton, 1947). In the 2-alkylthiobenzthiazoles and the isomeric *N*-alkyl compounds, on the contrary, donation of electrons would appear to be the characteristic function leading to chemical reactivity, for they readily give addition compounds with bromine and with copper salts. Much more drastic experimental conditions are required for other types of reactions, for example, hydrolysis, requiring electron accession to the carbon atom. As already mentioned, the cyclic type of compound (III) is believed to act through conversion to the aryl thiocarbimide which in turn reacts with a cell constituent. Although there is no conclusive evidence for this breakdown *in vivo*, the conception is supported by the finding (hitherto unpublished) that the fungistatic activity of compounds of type (III), in which substituents in the aryl nucleus are varied, exactly parallels that of the corresponding thiocarbimides (Davies & Sexton, 1946).

It is to be noted that the interchange of oxygen and sulphur in the carbamates (Table 5) has minor effects which might well be ascribed to no more than the differences in physical properties. The same absence of major effect on substitution of oxygen for sulphur was observed in the fungistatic activities of these substances (Davies & Sexton, 1946).

SUMMARY

1. The toxicities by contact and by stomach action of organic sulphur compounds of the following classes to various insects are recorded: benzthiazole derivatives and related quinoline and oxazole compounds, thio- and dithio-carbamates, substituted thiadiazines ('carbothialdines').

2. There is a marked selectivity in the action of the various substances against different insect species. Marked contact aphicidal activity is shown by 2-thio-3-methylbenzthiazolone (II, $R=CH_3$) and high toxicity to locusts (stomach poison) by the thiadiazine (III) ($R=CH_3$, $R'=C_6H_5$).

3. The results as a whole are discussed in relation to the hypothesis of Läuger, Martin & Müller (1944) which relates contact insecticidal action to the presence in the molecule of a 'toxic' group and a 'carrier' group, and it is concluded that the physical properties of the molecules concerned play a dominating part in deciding their insecticidal activity.

We are indebted to Prof. A. Robertson of Liverpool University and Dr F. P. Reed for the original preparations of 2-alkylthiobenzthiazoles and the preparation of the quinoline derivatives. Certain other compounds were prepared as described in papers referred to in the text by Dr A. D. Ainley, Dr H. Gudgeon and Dr J. C. Harland. The emulsions were prepared by Dr B. Collie. We wish to make acknowledgement also to the entomologists at Hawthorndale Laboratories, Jealott's Hill Research Station, where the biological testing methods were devised and the routine evaluations carried out, and particularly to Messrs M. D. Price and F. J. D. Thomas who were associated with this work from its commencement.

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Chemical Constitution and Insecticidal Action

2. SUBSTITUTED α-AMINONITRILES

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(*Received* 5 *March* 1948)

The high contact insecticidal activity of certain thiocyanic esters of alcohols of appropriate molecular weight and stereochemical composition prompted the investigation of other compounds containing the cyano group. So far as is known, the insecticidal activity of nitriles is markedly less than that of the corresponding thiocyanates. From our own observations, for example, lauronitrile has only a fraction of the aphicidal activity of dodecyl thiocyanate. If, as has been suggested by Hoggarth & Sexton (1947), the insecticidal activity of the thiocyanates is in part due to the special chemical reactivity of the carbon atom in the SCN group, then it follows that the introduction of other groups which modify the reactivity of the C atom of the CN group in a similar way may be expected to give compounds having insecticidal properties.

The cyanamides were considered since they, like the thiocyanates, have the CN group separated from the rest of the molecule by a heteroatom, but an investigation with Dr W. H. Davies showed that their high chemical reactivity (notably their tendency to polymerization) rendered them unattractive for the purpose in view. The separation of the nitrogen atom from the CN group gives the α-aminonitriles, a class of compound which is readily prepared with a variety of structural permutations. Although these have additional reactive features (see Discussion, p. 472) the CN group can be hydrolyzed under appropriate experimental conditions, as can the CN group in the thiocyanates. It was, therefore, decided to conduct a systematic investigation into their insecticidal activity. Some very powerful insecticides were thereby discovered from amongst the many dozens of compounds examined (see Collie, Hill & Sexton, 1939; Sexton, 1939).

Our work was interrupted by the war, and since then the appearance of the newer chlorinated hydrocarbon contact insecticides has not encouraged us to reopen the investigation. Nevertheless, points of considerable theoretical interest emerged, and this communication records such of the results as have a bearing on the problem of correlating constitution with activity. Since our work was carried out, certain compounds of this broad chemical class have been found by Siegler & Gertler (1946) to be toxic to codling moth larvae (*Cydia pomonella* L.), but so far as we are aware they have not been mentioned in any other scientific publication.

METHODS

Preparation of compounds

The general preparative methods are based upon the principle of condensing an aldehyde or ketone cyanohydrin with NH_3 or a primary or secondary amine, and four variations of experimental technique were employed (Jay & Curtius, 1894; Knoevenagel, 1904; Kötz & Merkel, 1926; Walther & Hübner, 1916). Trimeric methyleneaminoacetonitrile ('α-hydroformamine cyanide') was obtained by the 'literature' method (*Organic Syntheses*, Coll. Vol. 1, 348; Johnson & Rinehart, 1924). Many of the other compounds had already been described in the literature, but some were new, and where these had marked biological activity, their identity was checked by elementary analysis. Details of the preparation of three of the most important of the new compounds are appended, and Table 1 gives the properties of all the new compounds.

α-Phenylmethylaminooctoic nitrile, $C_6H_5(CH_3)N.CH(C_6H_{13})CN$.

Purified heptaldehyde (405 ml.) was added during 30 min. with stirring to 40% $NaHSO_3$ solution (615 ml.). The hot solution was then treated with methylaniline (360 ml.) during 20 min., during which time the reaction mixture assumed a jelly-like consistency. After 2·5 hr. at 70–75° the mass became transparent and fluid. A solution of 90% NaCN (180 g.) in water (360 ml.) was then added during 20 min., the temperature being kept below 70°. Stirring was continued for a further 20 min., and after addition of 1 l. of water, the supernatant oil was separated, washed and dried. Yield, 567 g. (82% of theory on heptaldehyde). The material had b.p. 156–157°/2 mm., and when freshly distilled was a white fluorescent oil. On storage it gradually turns yellow. (Found: C, 78·85; H, 10·0; N, 11·7. $C_{15}H_{22}N_2$ requires C, 78·3; H, 9·6; N, 12·1%.)

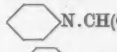
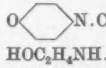
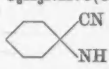
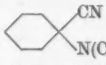
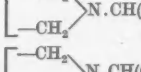
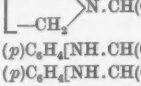
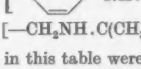
αβ-Bis(α'-cyanoheptylamino)ethane, $[—CH_2NH.CH(C_6H_{13})CN]_2$.

Purified heptaldehyde (134 ml.) was added to 40% $NaHSO_3$ solution (310 ml.). The solution was maintained at 60° for 30 min., when 90% ethylenediamine (37 ml.) was added. After 2 hr. stirring at 60°, a solution of 90% NaCN (56 g.) in water (172 ml.) was run in (30 min.), and after a further 30 min. the oil was separated and washed first with brine, then twice with water. It was dried over $MgSO_4$ and slowly solidified, giving white plates (from light petroleum), m.p. 45°. (Found: C, 70·3; H, 10·6; N, 18·1. $C_{18}H_{34}N_4$

requires C, 70·6; H, 11·1; N, 18·3%.) Treatment in glacial acetic acid with $NaNO_2$ gave the *dinitroso* derivative, m.p. 84–87°. (Found: N, 22·6. $C_{18}H_{32}O_2N_6$ requires N, 23·1%.)

As originally prepared, this substance was an oil, and it was only when further preparations were made that it was eventually obtained in the solid state. Solidification of the oil is sometimes extremely slow, and the earlier tests were performed with liquid preparations. Biological tests showed, however, that there was practically no difference in aphicidal activity between the original liquid preparations and the pure solid, and the original preparations are considered to consist substantially of the desired material. Heptaldehyde cyanohydrin, a possible contaminant in the liquid material, was not aphicidal.

Table 1. *Preparation and properties of new aminonitriles*

No.	Formula	Physical constants
23	$C_6H_5NH.CH(CH_3)CN$	M.p. 90–91°
27	$HOC_2H_4NH.CH(C_3H_7)CN$*	B.p. 126–128°/20 mm.
24	$C_6H_5(CH_3)N.CH(C_3H_7)CN$	B.p. 162–164°/15 mm.
20	$C_6H_5(C_2H_5)N.CH(C_3H_7)CN$	B.p. 155–156°/15 mm.
29	$HOC_2H_4NH.CH(C_6H_5)CN$	B.p. 160°/16 mm.
11	$(CH_3)_2N.CH(C_6H_{13})CN$	B.p. 120°/16 mm.
12	$(C_4H_9)_2N.CH(C_6H_{13})CN$	B.p. 171°/17 mm.
16	$(C_5H_{11})_2N.CH(C_6H_{13})CN$	B.p. 185–190°/18 mm.
30	$C_5H_{11}NH.CH(C_6H_{13})CN$	B.p. 170°/18 mm.
31	*cyclo*$C_6H_{11}NH.CH(C_6H_{13})CN$	B.p. 150°/16 mm.
15		B.p. 156°/16 mm.
15*a*		Low m.p., b.p. 166°/16 mm.
28	$HOC_2H_4NH.CH(C_6H_{13})CN$	M.p. 78–80°
26	$C_6H_5NH.CH(C_6H_{13})CN$	B.p. 170°/17 mm.
17	$C_6H_5(CH_3)N.CH(C_6H_{13})CN$	B.p. 156–157°/2 mm.
19	$C_6H_5(C_2H_5)N.CH(C_6H_{13})CN$	B.p. 192–194°/18 mm.
36	*cyclo*$C_6H_{11}NH.C(CH_3)_2CN$	M.p. 52–53°
37	$HOC_2H_4NH.C(CH_3)_2CN$	B.p. 101–103°/27 mm.
42	$C_6H_5NH.C(CH_3)[CH_2.CH.(CH_3)_2].CN$	M.p. 52–53°
45		M.p. 72–73°
47*a*		Liquid
10	$(C_2H_5)_2N.CH(C_{17}H_{35})CN$	Waxy solid
63	$[—CH_2NH.C(CH_3)(C_2H_5)CN]_2$	M.p. 46–47°
53	$[—CH_2N(CH_3).CH(C_6H_{13})CN]_2$	M.p. 48–51°
57		M.p. 165°
58		M.p. 149–151°
59	$(p)C_6H_4[NH.CH(C_3H_7)CN]_2$	M.p. 116–118°
60	$(p)C_6H_4[NH.CH(C_6H_{13})CN]_2$	M.p. 145°
51	$[—CH_2CH_2CH_2NH.CH(C_3H_7)CN]_2$	Undistillable liquid
52	$[—CH_2CH_2CH_2NH.CH(C_6H_{13})CN]_2$	Undistillable liquid
61		M.p. 182–184°
54	$[—CH_2NH.C(CH_3)(C_{11}H_{23})CN]_2$	Undistillable liquid

The new compounds listed in this table were not subjected to elementary analysis since (*a*) they were all of the same chemical type, prepared by standardized methods, and (*b*) their physical properties were in accord with expectations from the properties of near homologues, which have been fully characterized and described in the literature. For some of the more important new compounds, analyses are appended to the detailed preparative descriptions given on pp. 468–470.

* C_3H_7, C_4H_9, C_5H_{11}, etc. indicate normal primary straight-chain alkyl radicals unless otherwise stated.

αβ-Bis(N-methyl-α'-cyanoheptylamino)ethane, $[—CH_2N(CH_3)CH(C_6H_{13})CN]_2$. Redistilled heptaldehyde (22·8 g.) was slowly run into 40% $NaHSO_3$ (57·2 ml.) at 60–70°. After 30 min. a solution of *NN'*-dimethylethylenediamine dihydrochloride (16·1 g.) in 2N-NaOH (100 ml.) was added. After further stirring (1 hr.) at the same temperature, a solution of NaCN (12 g.) in water (18 ml.) was added. The oil which separated was washed with brine, and dried with $MgSO_4$; yield, 32·8 g. (94% of theory). It could not be distilled without decomposition, but it solidified slowly and had m.p. 48–51° (from methanol). (Found: C, 71·95; H, 10·8; N, 17·3. $C_{29}H_{58}N_4$ requires C, 71·8; H, 11·4; N, 16·8%.)

Biological testing

Sorting tests were carried out using aphids (usually the chrysanthemum aphis, *Macrosiphoniella sanborni* G., but the bean aphis, *Doralis rumicis* L. (=*Aphis rumicis* L., (*fabae* Scop.)), when the former were not available) and adult blowflies (*Calliphora erythrocephala* Meig.) with contact spraying in the Tattersfield apparatus, and for stomach poisoning effects using the grain weevil, *Sitophilus granarius* L., which was allowed to feed on 'biscuits' prepared from flour and water containing the substance under test. These methods, including the methods used for the preparation of the sprays, are described by Davies & Sexton (1948); in brief, the dispersions for spraying were made by adding ethanolic solutions of the substances under test to a dilute solution of a dispersing agent, so that the final sprays contained 50% ethanol. The possible interfering effect of the ethanol was appreciated, and the activity of promising substances was confirmed by the examination of specially prepared dispersions. The ethanol dispersion technique served to provide a standard method of presentation, applicable to most of the compounds examined, and so to permit comparisons of activity in groups of closely related compounds. Cases where this dispersion technique was not applicable are noted in the tables. In the stomach poison tests, lead arsenate was used as a standard of comparison, and the figures in brackets in Table 2 are the toxicities of lead arsenate in the individual experiments. Toxicities are expressed as 'absolute mortality', a figure derived after adjustment for mortalities caused by sprays or 'biscuits' from which the poison was omitted (Davies & Sexton, 1948).

RESULTS

The earliest tests were stomach poison tests, and, using the 'biscuit' method, α-hydroformamine cyanide was found to be highly toxic not only to the grain weevil, but also to the flour moth, *Ephestia kühniella* Zell., and moderately toxic to leatherjackets. It was without effect on rice moth larvae (*Corcyra cephalonica* Staint.). Iminodiacetonitrile, $NH(CH_2CN)_2$, was also toxic to the grain weevil, but the methylene derivative of this, $CH_2[N(CH_2CN)_2]_2$, was non-toxic. It was these results which led to the decision to conduct a systematic investigation into the α-aminonitriles. The results of the sorting tests are summarized in Table 2.

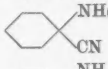
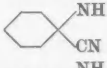
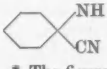
The results of the above preliminary evaluation showed that high aphicidal activity could be obtained with compounds of the aminonitrile class. The next stage of the investigation was, therefore, a further examination of the more promising compounds at greater dilutions, and also an extension of the investigation to compounds derived from diamines. In selecting compounds for further study some account was taken of their potential availability. The results of the further sorting tests, using the standard ethanol dispersion method on chrysanthemum aphis in the Tattersfield apparatus, are given in Table 3.

Since most of the figures for mortality in Table 3 had been obtained by using the 50% ethanol sprays, it was necessary to make compositions which could be diluted with water to provide sprays which were essentially aqueous in nature, though the presence of a surface-active agent as emulsifier was of course necessary. For this purpose concentrated emulsions of the following composition by weight were prepared and were diluted either with water or with 0·1% 'Agral 2' (wetting agent) and sprayed on chrysanthemum aphis using the Tattersfield apparatus.

1. $C_6H_5(CH_3)N.CH(C_6H_{13})CN$ (Compound 17) 54%; ethanolamine oleate 36%; ethoxyethanol 10%.
2. $[—CH_2NH.CH(C_3H_7)CN]_2$ (Compound 49) 15·6%; sulphonated cod oil 38·3%; ethoxyethanol 46·1%.
3. Compound 49, 20%; 'Agral' Liquid Wetter 43%; ethoxyethanol 37%.
4. Compound 49, 8·3%; castor oil 16·7%; trichloroethylene 47%; casein 4·5%; borax 2·1%; water 64·5%.
5. Compound 49, 9·1%; triethanolamine oleate 89·1%; ethoxyethanol 1%.
6. $[—CH_2NH.CH(C_6H_{13})CN]_2$ (Compound 50) 20%; 'Agral' Liquid Wetter 43%; ethoxyethanol 37%.
7. Compound 50, 15·6%; sulphonated cod oil 38·3%; ethoxyethanol 46·1%.
8. Compound 50, 10%; castor oil 10%; trichloroethylene 45%; casein 4·3%; borax 2·1%; water 64·5%.

The results of spraying tests with the above emulsions at 0·05% active ingredient are given in Table 4. Clearly, although the degree of activity was considerably affected by the nature of the emulsion, the high aphicidal activity revealed by the sorting test was genuine. This was further confirmed by trials of dry dust preparations (3% on talc) against bean and hop aphis, *Phorodon pruni* Scop., on infested plants, with nicotine and 2-thio-3-methylbenzthiazolone (Davies & Sexton, 1948) included for comparison. The results are given in Table 5. Possibly the superiority of nicotine in this experiment was due to its fumigant action. It may be mentioned at this point that, in experiments on infested plants, marked variations in phytocidal activity between the different emulsions were also noted, and that the most effective emulsions from the aphicidal point of view were not necessarily the most phytocidal.

Table 2. *Insecticidal sorting tests with (mono)α-aminonitriles*

No.	Compound	Percentage absolute mortality: Aphids Conc. (%) 0·35	Aphids 0·2	Blowflies 0·55	Blowflies 0·35	Grain weevils ('biscuit' method)
1	$N(CH_2CN)_3$	28	—	—	17	—
2	$(CH_3)_2N.CH_2CN$	—	3 (*b*)	50	—	22 (58)*
3	$(C_2H_5)_2N.CH_2CN$	—	6 (*b*)	69	—	8 (58)
4	$(C_4H_9)_2NCH_2CN$	—	3 (*b*)	97	—	57 (58)
5		—	3 (*b*)	85	—	10 (58)
6	$C_6H_5(CH_3)N.CH_2CN$	—	3 (*b*)	27	—	83 (47)
7	$(C_2H_5)_2N.CH(CH_3)CN$	—	28 (*b*)	17	—	8 (92)
8	$(C_2H_5)_2N.CH(C_6H_{13})CN$	—	88 (*b*)	99	—	88 (44)
9	$(C_2H_5)_2N.CH(C_6H_5)CN$	—	34 (*b*)	100	—	99 (44)
10	$(C_2H_5)_2N.CH(C_{17}H_{35})CN$	97	18†	—	4	—
11	$(CH_3)_2N.CH(C_6H_{13})CN$	77	—	92	—	78 (50)
12	$(C_4H_9)_2N.CH(C_6H_{13})CN$	100	—	100	—	16 (50)
13	$(C_4H_9)_2N.CH(CH{:}CH.CH_3)CN$	100	—	—	18	—
14	$(HOC_2H_4)_2N.CH(C_6H_{13})CN$	41, 25†	—	—	3, 6†	—
15		100	—	100	—	54 (50)
15*a*		90†	—	58†	—	—
16	$(C_5H_{11})_2N.CH(C_6H_{13})CN$	100†	—	—	19†	—
17	$C_6H_5(CH_3)N.CH(C_6H_{13})CN$	100	—	—	19	3 (50)
18	*cyclo*$C_6H_{11}(C_2H_5)N.CH(C_6H_{13})CN$	100	—	—	—	20 (95)
19	$C_6H_5(C_2H_5)N.CH(C_6H_{13})CN$	28†	—	—	58†	—
20	$C_6H_5(C_2H_5)N.CH(C_2H_7)CN$	53, 4†	—	—	96, 81†	56 (95)
22	$H(C_6H_5)N.CH_2CN$	—	17 (*b*)	31	—	90 (45)
23	$H(C_6H_5)N.CH(CH_3)CN$	37	—	—	4	21 (42)
24	$C_6H_5(CH_3)N.CH(C_3H_7)CN$	64†	—	—	54†	12 (41)
25	$H(C_6H_5)N.CH(C_3H_7)CN$	27	—	—	37	10 (41)
26	$H(C_6H_5)N.CH(C_6H_{13})CN$	54	—	59	—	8 (50)
27	$HOC_2H_4NH.CH(C_3H_7)CN$	56	—	—	9	9 (95)
28	$HOC_2H_4NH.CH(C_6H_{13})CN$	100	100	22	—	2 (59)
29	$HOC_2H_4NH.CH(C_6H_5)CN$	39	—	16	—	11 (59)
31	*cyclo*$C_6H_{11}NH.CH(C_6H_{13})CN$	100	—	99	—	20 (56)
35	$H(C_6H_5)N.C(CH_3)_2CN$	54	—	—	48	8 (41)
40	$H(C_6H_5)N.C(CH_3)(C_2H_5)CN$	86	71†	—	65	5 (41)
42	$H(C_6H_5)N.C(CH_3)(C_4H_9\beta)CN$	97	39†	—	28	6 (41)
44		100	60†	—	10	5 (51)
45		0	—	—	26	0 (95)
46		82	20†	—	11	0 (42)

* The figures in brackets in this column give the mortality with a lead arsenate bait.
† Emulsified by means of a sulphonated oil.
The chrysanthemum aphis was used except where (*b*) indicates the bean aphis.

In addition to the compounds listed above, the following gave absolute mortalities of not more than 15% to all three insects:

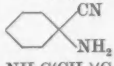
No.	Compound	No.	Compound
21	$CH_3NH.CH_2CN$	37	$HOC_2H_4NH.C(CH_3)_2CN$
30	$C_5H_{11}NH.CH(C_6H_{13})CN$	38	$NH_2C(CH_3)(C_2H_5)CN$
32	Benzoyl derivative of 31	39	Oxalate and tartrate of 38
33	$NH_2C(CH_3)_2CN$	43	$HOC_2H_4NH.C(CH_3)(C_4H_9\beta)CN$
34	Four acyl derivatives of 33	47	
36	*cyclo*$C_6H_{11}NH.C(CH_3)_2CN$	41	$NH_2C(CH_3)(C_4H_9\beta)CN$

Table 3. *Further aphicidal sorting tests with aminonitriles*

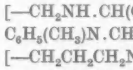
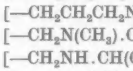
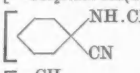
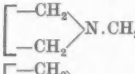
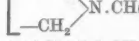
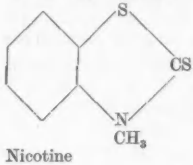
No.	Compound	Percentage absolute mortality of chrysanthemum aphis			
	Conc. (%) ...	0·35	0·2	0·05	0·025
48	$[—CH_2NH.CH(CH_3)CN]_2$	100	98	40	—
49	$[—CH_2NH.CH(C_2H_7)CN]_2$	100	100	96	23
50	$[—CH_2NH.CH(C_6H_{13})CN]_2$	100	100	100	98
17	$C_6H_5(CH_3)N.CH(C_6H_{13})CN$	100	80	35	—
51	$[—CH_2CH_2CH_2NH.CH(C_2H_7)CN]_2$	100	100	34	—
52	$[—CH_2CH_2CH_2NH.CH(C_6H_{13})CN]_2$	100	100	89	—
53	$[—CH_2N(CH_3).CH(C_6H_{13})CN]_2$	100	100	98	90
54	$[—CH_2NH.CH(CH_3)(C_{11}H_{23})CN]_2$	90	26	16	—
55		21	—	—	—
56		0	—	—	—
57		100	—	—	—
58		0	—	—	—
59	$(p)C_6H_4[NH.CH(C_3H_7)CN]_2$	8	—	—	—
60	$(p)C_6H_4[NH.CH(C_6H_{13})CN]_2$	11	—	—	—
61	$[—(1{:}4\text{-}cyclo)C_6H_{10}NH.CH(C_3H_7)CN]_2$	18	—	—	—
62	$[—CH_2NH.C(CH_3)_2CN]_2$	26	—	—	—
63	$[—CH_2NH.C(CH_3)(C_2H_5)CN]_2$	48	—	—	—
64	$[—CH_2NH.CH(C_6H_5)CN]_2$	8	—	—	—
12	$(C_4H_9)_2N.CH(C_6H_{13})CN$	100	95	58	—
15		—	100	77	43
31	$cyclo C_6H_{11}NH.CH(C_6H_{13})CN$	—	100	84	13
28	$HOC_2H_4NH.CH(C_6H_{13})CN$	—	100	58	—
13	$(C_4H_9)_2N.CH(CH{:}CH.CH_3)CN$	—	100	25	—
18	$cyclo C_6H_{11}(C_2H_5)NCH(C_6H_{13})CN$	—	100	72	—
—	$C_{12}H_{25}SCN$	100	100	100	63

Table 4. *Toxicity of aminonitrile emulsions to chrysanthemum aphis with a concentration of 0·05% insecticide*

Emulsion	Percentage absolute mortality	
	Diluted with water	Diluted with 0·1% 'Agral 2'
1	100	—
Spray of active ingredient in 50% ethanol	49	—
2	37·5	—
3	25	—
4	69	44
5	58	47
Spray of active ingredient in 50% ethanol	62	—
6	96	98
7	98	60
8	100	100
Spray of active ingredient in 50% ethanol	100	—

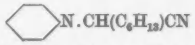
Table 5. *Toxicity of 3% dusts to aphis*

Insecticide	Percentage kill	
	Bean aphis	Hop aphis
$[—CH_2NH.CH(C_6H_{13})CN]_2$	39	22
$C_6H_5(CH_3)N.CH(C_6H_{13})CN$	63	47
	48	2
Nicotine	98	97·5

DISCUSSION

The first point which emerges from a study of the above results is the variation in toxicity to different insect species, an effect which was observed in

studies of organic sulphur compounds and which, indeed, is probably a general feature of organic insecticides (Davies & Sexton, 1948). Certain of the more potent insecticides (Table 2) showed high toxicity to all three test insects, but the toxicity was specific where evaluation at lower dosages was carried out. For example, the two compounds



and $C_6H_{11}NH.CH(C_6H_{12})CN$,

when tested at 0·2 %, gave 100 % absolute mortality of aphids but were non-toxic to blowflies. Again, extended evaluation of compounds having marked stomach-poison activity against the grain weevil showed that they were much inferior to lead arsenate against various leaf-eating larvae. This was true, for example, with $(C_2H_5)_2N.CH(C_6H_5)CN$ (Table 2) which, though more toxic than lead arsenate to the grain weevil, was practically without action against a wide range of leaf-eating larvae. The specificity of α-hydroformamine cyanide as a stomach poison has already been referred to. We found phenylglycine nitrile to be more toxic than lead arsenate to the grain weevil. Siegler & Gertler (1946) found it to be less toxic than lead arsenate to the codling moth, but they found the *p*-tolyl analogue to be more toxic than lead arsenate to the codling moth.

Considering the contact insecticidal action and particularly the effects against aphis, it is clear that the physical properties of the molecule play an important part in influencing its toxicity. Consideration of Tables 2 and 3 shows numerous instances of this, including the effects of ascending an homologous series (cf. the results of Bousquet, Salzberg & Dietz, 1935, with thiocyanates). A further illustration of the importance of physical properties is provided by the effect of introducing an aliphatic hydroxyl group. This will tend to decrease the lipid/water partition, a property which is of profound importance in certain narcotics, and which may be equally relevant in contact insecticides. If this is so, it is to be expected that the introduction of a hydroxyl group may produce changes as great as the move of several steps in an homologous series. A good instance of this is seen in the comparison of compounds 8 and 14 (Table 2). Of the compounds containing aliphatic OH, only one (no. 28, Tables 2 and 3) showed marked aphicidal activity. Clearly, therefore, the inclusion of such groups is not necessarily deleterious, but until more is known of the precise relationships of water and lipid solubility with insecticidal activity, no predictions are possible. Another physicochemical factor of apparent significance is that of molecular shape. This is illustrated by the comparison of two pairs of compounds, 30 with 46 and 51 with 55. The molecular shape may be expected to have a profound effect on physical properties, and thus to influence such factors as lipid/water partition and membrane permeability.

As regards the chemical reactivity of the aminonitriles, their behaviour on hydrolysis is of possible significance. This can take two pathways:

$$R_2N.CR_2CN \begin{cases} \rightarrow R_2NH + R_2CO + HCN, \\ \rightarrow R_2N.CR_2CONH_2 \rightarrow R_2N.CR_2COOH, \end{cases}$$

and liberation of cyanide in the biophase must be considered as having a possible bearing on their mode of action. We do not believe, however, that this is the ultimate mechanism of the insecticidal activity for the following reasons. Phenylmethylaminooctoic nitrile (no. 17) causes 80 % absolute mortality of chrysanthemum aphis at 0·2 %. This is equivalent to a concentration of approximately 0·02 % HCN. With compound no. 50, producing 98 % absolute mortality at 0·025 %, the equivalent HCN concentration would be only 0·004 %. Actual experiment showed that sodium cyanide was practically non-toxic to chrysanthemum aphis at 0·04 %. This evidence is reinforced by the lack of aphicidal activity in heptaldehyde cyanohydrin, a fat-soluble compound which can also give rise to cyanide on decomposition.

It is our view that the insecticidal activity of the α-aminonitriles discussed in this paper is to be attributed partly to their chemical reactivity which enables them to become attached to cell constituents. This may be through the unsaturated nature of the cyano group, as in thiocyanates. Alternatively, it may be due to a displacement reaction whereby some group such as NH_2 in the cell displaces the group R_2N from the aminonitrile. We have, indeed, observed that heating $(C_2H_5)_2N.CH(C_6H_5)CN$ with aniline causes evolution of diethylamine. Superimposed on this reactivity and indeed dominating the entire situation, is the effect of the physicochemical properties of the molecule as a whole, the correct adjustment of such factors as lipid/water partition, molecular shape, surface activity, etc., determining the absorption of the insecticide and its distribution within the organism. This parallels the case of the thiocyanates, and conforms to the general views regarding contact insecticidal action put forward by Läuger, Martin & Müller (1944) and further illustrated by Davies & Sexton (1948).

SUMMARY

1. The insecticidal properties of a series of substituted α-aminonitriles have been examined, and marked activity found in certain compounds.

2. There is marked specificity in the resistance of different insects to individual compounds.

3. α-Phenylmethylaminooctoic nitrile,

$$C_6H_5(CH_3)N.CH(C_6H_{13})CN,$$

and αβ-bis(α′-cyano-α-heptylamino)ethane,

$$[—CH_2NH.CH(C_6H_{13})CN]_2,$$

have aphicidal activity only slightly less than that of dodecyl thiocyanate or nicotine.

4. The mode of action of these insecticides is discussed in relation to their chemical reactivity and their physicochemical properties.

We wish to thank the entomologists of Hawthornedale Laboratories, Jealott's Hill Research Station, and particularly Mr F. J. D. Thomas, for carrying out the biological testing. We are indebted also to Dr B. Collie for the preparation of emulsions.

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The Occurrence of Phenolic Substances in Arthropods

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Pryor (1940) showed that the hardening of the protein component of insect cuticle is due to the tanning action of an agent produced by oxidation of a phenolic substance. In the analogous hardening of the cockroach ootheca, Pryor, Russell & Todd (1946) showed that the phenolic substance concerned is 3:4-dihydroxybenzoic acid (I) and suggested a possible mechanism for the hardening process. It was clearly desirable to examine a number of insect species in order to establish the range of phenolic substances which might be concerned.

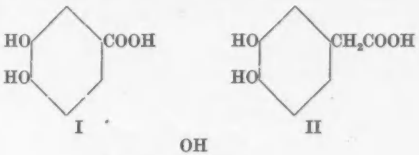
Polyhydric phenols appear to be widely distributed in arthropods, although very few have actually been isolated, most investigators having simply demonstrated their presence in ethanolic extracts by means of colour reactions. In this way, Pryor (1940) has shown that *o*-dihydric phenols occur in representatives of six orders of insects (Orthoptera, Odonata, Coleoptera, Hemiptera, Lepidoptera, Hymenoptera), and Lafon (1943) has found them in insects and in Arachnida (Scorpionidea, Araneida). In insects, phenols are associated with some of the layers of the epicuticle (Wigglesworth, 1947; Webb, 1947) and with the brown proteins (sclerotins) which are responsible for the hardness of the exocuticle. They also occur in the epicuticle of arthropods, whose exocuticle is hardened with calcium carbonate; by a less specific test (the reduction of ammoniacal silver oxide, performed on paraffin sections) we have shown that they are probably present in the epicuticle of a typical diplopod (*Tachypodoiulus niger* Leach), and Dennell (1947*b*) has found them in the epicuticle of several species of Crustacea Malacostraca. Using the same test, Beament (1947) has found that some of the layers of the chorion of the eggs of *Rhodnius prolixus* Stähl (Hemiptera; Reduviidae) are rich in dihydric phenols.

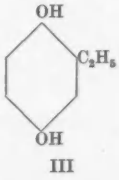
The earliest recorded isolation of a phenolic substance from insects is that of Villon, who is reported by Slater (1887) as having obtained 15 g. of an 'alcohol soluble tannin' from 500 g. of *Sitophilus granarius* L. Schmalfuss & Müller (1927) isolated 3:4-dihydroxyphenylalanine from the elytra of two species of *Melolontha*. Schmalfuss, Heider & Winkelmann (1933) isolated 3:4-dihydroxyphenylacetic acid (II) from the elytra of *Tenebrio molitor* L., and the same acid was obtained by Schmalfuss (1937) from the cuticles of *Cetonia aurata* L., *Potosia cuprea* F. and *Melolontha hippocastani* F. (Coleoptera; Scarabaeidae). More recently, Pryor *et al.* (1946) isolated 3:4-dihydroxybenzoic acid (protocatechuic

acid) (I) from oothecae of *Blatta orientalis*, and the same authors (Pryor *et al.* 1947) obtained 3:4-dihydroxybenzoic acid from puparia of *Calliphora erythrocephala* L. and 3:4-dihydroxyphenylacetic acid and α-hydroxy-β-(3:4-dihydroxyphenyl)-propionic acid (3:4-dihydroxyphenyllactic acid) from imagines of *Tenebrio molitor* L.

We have now examined the following species: *Locusta migratoria* L., *Rhizopertha dominica* F. (Coleoptera; Bostrychidae), *Sitophilus* (=Calandra auct. non Clairv.) *granarius* L., *S. oryzae* L. (Coleoptera; Curculionidae), *Tribolium confusum* J. du Val. and *T. ferrugineum* F. (=*castaneum* Herbst.) (Coleoptera; Tenebrionidae).

The experimental procedure was a modified version of that used in our previous studies and gave much higher yields of phenolic acids. The modifications included freeze drying of the insect material, followed by mechanical grinding, as well as minor changes in the methods of purification. During the isolation procedure, the phenolic acids present were methylated to facilitate working up, so that the acids were actually isolated as their methyl ethers. Previous work (Pryor *et al.* 1946, 1947) leaves no reasonable doubt that the acids present in the insects are in the unmethylated form. The results, which are recorded in the experimental portion, show that all the species examined contain 3:4-dihydroxyphenylacetic acid (II) and the *Tribolium* species contain, in addition, 3:4-dihydroxybenzoic acid (I). The low yield of 3:4-dimethoxyphenylacetic acid obtained from *Locusta* is perhaps to be ascribed to the low ratio of cuticle weight to total weight, as compared with the ratio in the other insects investigated. In addition to the insects mentioned in the experimental section we also re-examined imagines of *Tenebrio molitor*, but, in confirmation of our earlier results (Pryor *et al.* 1947), we were unable to detect in them any 3:4-dihydroxybenzoic acid.





III

Summarizing the information available from the present and earlier work, it appears that phenolic acids have been identified by isolation (as such or as their methyl ethers) from thirteen representative species belonging to three orders of Insecta, whilst colour reactions indicate the presence of similar *o*-dihydroxyphenolic substances in four additional orders. We feel justified, therefore, in assuming that these phenolic acids are of general occurrence and may all be concerned in cuticle hardening. Although the presence of traces of acids other than those identified cannot be wholly excluded, the pure methylated substances isolated by us accounted for more than 75% of the crude methylated phenolic extracts, so that the distribution of the identified acids is of some interest. Of the thirteen species so far examined, ten contained 3:4-dihydroxyphenylacetic acid, which in seven cases was the only acid present; in two cases it was accompanied by 3:4-dihydroxybenzoic acid, and in one by α-hydroxy-β-(3:4-dihydroxyphenyl)-propionic acid. 3:4-Dihydroxybenzoic acid occurred alone in two species. In addition, 3:4-dihydroxyphenylalanine has been isolated from two species. The most likely mode of participation of such *o*-dihydroxy aromatic acids in cuticle hardening is enzymic oxidation followed by condensation of the oxidized material with free amino groups in the cuticular protein, so that stable cross-linked structures are formed in which the nitrogen of the amino groups becomes directly attached to the aromatic nuclei. Thiol groups in the protein may, of course, also take part in reaction with the oxidized dihydroxy acid. The process, indeed, bears a close resemblance to the tanning of leather with quinones. Catechol and 3:4-dihydroxybenzoic acid and their homologues when oxidized condense *in vitro* with compounds containing free amino groups to give compounds in which nitrogen is directly attached to the aromatic nucleus. Reactions of this type are being studied at present as an aspect of the work here described and will be reported upon in due course. As to the origin of the phenolic acids in insects, the most probable explanation is that they are degradation products of 3:4-dihydroxyphenylalanine, itself produced from tyrosine under the influence of a polyphenol oxidase. It is of interest to note that both Fraenkel & Rudall (1947) and Dennell (1947*a*) have also expressed the view that the phenol hardening in blowfly puparia is brought about by a phenolic material produced from blood tyrosine by enzymic oxidation.

Since it was desired to make our investigations as comprehensive as possible, the solutions of sulphurous acid containing cyanide in which the insects were killed and stored were always examined for the presence of phenolic substances. In the case of the two *Tribolium* species, but in no others, ether extracts of the acidic liquor, after removal of the dead insects, yielded large quantities of ethylhydroquinone (1·4% on freeze-dried insect material) identified by analysis, mixed melting point and preparation of the *dibenzoate*. This substantially con-

firms the findings of Alexander & Barton (1943), who examined the volatile secretion of the flour beetles, *Tribolium confusum* J. du Val. and *T. ferrugineum* F. (= *castaneum* Herbst.), which causes flour infested by these beetles to become pink in colour and unpalatable; they concluded on the evidence available to them that the active principle was ethyl *p*-benzoquinone. Presumably in our experiments the insects, on being dropped into the sulphurous acid solution, eject ethyl *p*-benzoquinone which is then reduced to the hydroquinone. Quinones have previously been recorded in secretions of the stink glands of other arthropods. Béhal & Physalix (1900) described a compound with the properties of a *p*-quinone in the lateral stink glands of the millipede, *Schizophyllum mediterraneum* Latzel, but did not isolate it; similar observations on other diplopods are described by Burtt (1947). Ethyl *p*-benzoquinone is probably also the chief constituent of the yellow oils obtained by Palm (1946) from stink glands on the prothorax and on the ventral side of the tip of the abdomen of *Aphanotus destructor* Uytt. and *Tribolium confusum* J. du Val.

The crude crystalline material first obtained by us during the isolation of ethylhydroquinone had a low melting point and was difficult to purify. Careful fractionation of crystallization mother liquors obtained in purifying the ethylhydroquinone and its dibenzoate yielded products which, although melting lower than methylhydroquinone (toluhydroquinone) and its dibenzoate, showed no depression in melting point when mixed with authentic specimens of these materials, but were depressed in melting point by pure ethylhydroquinone and its dibenzoate. From these facts and the elementary analysis, we conclude that the extract contained small amounts of toluhydroquinone. It is probable that the similar difficulty experienced by Alexander & Barton (1943) in purifying their material was due to the presence in the volatile secretion of small amounts of methyl *p*-benzoquinone (toluquinone) as well as ethyl *p*-benzoquinone.

The most probable precursor of these *p*-quinones would seem to be 2:5-dihydroxyphenylalanine, an acid already suggested as an intermediate in the formation of homogentisic acid (2:5-dihydroxyphenylacetic acid) in human alcaptonuria (Neuberger, Rimington & Wilson, 1947). 2:5-Dihydroxyphenylalanine itself has not been found in nature, but its production from tyrosine (cf. Blaschko & Sloane Stanley, 1948) by oxidation as an alternative to 3:4-dihydroxyphenylalanine would seem reasonable enough. Such an oxidation would be analogous to the well-known formation of toluhydroquinone on oxidizing *p*-cresol with potassium persulphate in acid solution, rearrangement with migration of the methyl group occurring in the process (Kumogi & Wolffenstein, 1908); a similar migration of the aminopropionic acid side chain during the oxidation of tyrosine would yield 2:5-dihydroxyphenylalanine, which might then be degraded to ethylhydroquinone and toluhydroquinone. Wolkow & Baumann (1891) have already shown that the intestinal bacteria in dogs can convert homogentisic acid to toluhydroquinone.

EXPERIMENTAL

Methylated phenolic acids from insects

The insects were collected and stored in water containing KCN to inactivate polyphenol oxidases, and SO_2 to prevent aerial oxidation of hydroquinone or catechol derivatives. Before working up, the material (whole insects, except in the case of *Locusta* where only the heads were used) was separated from the liquid, freeze dried and ground in a mechanical mortar. It was then extracted with methanol in a vapour-jacketed Soxhlet for 24 hr., filtered, the methanolic extracts (which had a blue fluorescence) evaporated to dryness under reduced pressure in an atmosphere of N_2 and the residue extracted with water until the extracts gave a negative reaction with $FeCl_3$. The combined aqueous extracts were acidified to congo red with H_2SO_4, extracted continuously with peroxide-free ether, the ethereal extract concentrated to *c.* 100 ml. and the phenols present methylated by addition of ethereal diazomethane (from 20 g. nitrosomethylurea). The solution so obtained was left overnight, solvents removed by evaporation and the residual oil refluxed for 2 hr. with methanolic KOH (50 ml. of 2%) to hydrolyze ester groups. After removal of methanol, water was added, the solution acidified with H_2SO_4 and extracted with ether. The ethereal extract was shaken with aqueous Na_2CO_3 to remove acids, and the aqueous layer was again acidified and extracted with ether. The ethereal extract dried over Na_2SO_4 and evaporated yielded usually a semi-solid mass. Fractional crystallization, or, where only one acid was present, simple recrystallization, from a mixture of benzene and light petroleum yielded the pure methylated acids. The products obtained in each case are given below:

Locusta migratoria. 3:4-Dimethoxyphenylacetic acid, m.p. 97°, undepressed by an authentic specimen, m.p. 98° (Found: C, 61·3; H, 6·4. Calc. for $C_{10}H_{12}O_4$: C, 61·2; H, 6·2%). Yield, 0·2% on dried material (415 g.).

Rhizopertha dominica. 3:4-Dimethoxyphenylacetic acid, m.p. and mixed m.p. 98° (Found: C, 61·9; H, 6·0%). Yield, 1·5% on dried material (30 g.).

Sitophilus granarius. 3:4-Dimethoxyphenylacetic acid, m.p. and mixed m.p. 96° (Found: C, 61·3; H, 6·0%). Yield, 1·8% on dried material (101 g.).

S. oryzae. 3:4-Dimethoxyphenylacetic acid, m.p. and mixed m.p. 97° (Found: C, 60·9; H, 5·9%). Yield, 1·4% on dried material (128 g.).

Tribolium confusum. 3:4-Dimethoxyphenylacetic acid (yield, 1·4%), m.p. and mixed m.p. 96° (Found: C, 61·1; H, 6·4%), together with 3:4-dimethoxybenzoic acid (yield, 0·4%), m.p. 179°, undepressed by an authentic specimen, m.p. 180° (Found: C. 59·3; H, 5·7. Calc. for $C_9H_{10}O_4$: C, 59·3; H, 5·5%). The amount of dried insects used was 45 g.

T. ferrugineum. 3:4-Dimethoxyphenylacetic acid (yield, 1·9%), m.p. and mixed m.p. 98° (Found: C, 61·0; H, 5·9%),

together with 3:4-dimethoxybenzoic acid (yield, 0·5%), m.p. and mixed m.p. 180° (Found: C, 59·1; H, 5·6%). The amount of dried insects used was 57 g.

Isolation of ethylhydroquinone (1:4-*dihydroxy*-2-*ethylbenzene*)

The acidic liquid from the *Tribolium* species, after separation of the insect bodies (dry wt. 100 g.), was continuously extracted with ether for 24 hr., and the ethereal extract dried over Na_2SO_4 and evaporated. The residual oil was now treated with an excess of concentrated aqueous $FeCl_3$ solution and quinones formed were collected by steam distillation. The steam distillate was saturated with SO_2 and extracted with ether. Evaporation of the ethereal extract gave the crude ethylhydroquinone as a colourless crystalline solid, m.p. 106°, unaltered by repeated recrystallization from benzene or chloroform. After several recrystallizations from water (charcoal) the product formed colourless plates, m.p. 114°, alone or in admixture with authentic ethylhydroquinone (m.p. 114°) prepared by Clemmensen reduction of 2:5-dihydroxyacetophenone (Found: C, 69·9; H, 7·5. Calc. for $C_8H_{10}O_2$: C, 69·6; H, 7·3%). For further confirmation of its identity, the product was treated with excess of benzoyl chloride in pyridine. The dibenzoate so formed separated from methanol in colourless needles, m.p. 89°, undepressed in admixture with authentic dibenzoate of ethylhydroquinone (m.p. 89°) prepared as described below (Found: C, 76·1; H, 5·0. Calc. for $C_{22}H_{18}O_4$: C, 76·2; H, 5·2%).

Concentration of the aqueous crystallization mother liquors of the ethylhydroquinone yielded a crop of material which, after further fractionation from water and recrystallization from benzene, had m.p. 114°, undepressed in admixture with toluhydroquinone (m.p. 124°), but depressed in admixture with ethylhydroquinone (m.p. 114°). Similarly, fractional crystallization from methanol of the dibenzoate prepared from the crude ethylhydroquinone, yielded a small amount of material, m.p. 93°, undepressed in admixture with the dibenzoate of toluhydroquinone (m.p. 122°), but depressed in admixture with the dibenzoate of ethylhydroquinone (m.p. 89°) (Found: C, 76·1; H, 5·1. Calc. for $C_{21}H_{16}O_4$: C, 75·9; H, 4·85. Calc. for $C_{22}H_{18}O_4$: C, 76·2; H, 5·2%).

Ethylhydroquinone dibenzoate. Benzoyl chloride (1 ml.) was added to a solution of ethylhydroquinone (0·5 g.) in dry pyridine (10 ml.). Reaction set in at once and the mixture was allowed to stand overnight and then poured on a mixture of ice and dilute H_2SO_4 and extracted with ether. The ethereal extract was thoroughly washed, dried over Na_2SO_4 and evaporated. Recrystallization of the solid residue from methanol (charcoal) gave *ethylhydroquinone dibenzoate* as colourless needles, m.p. 89° (Found: C, 76·2; H, 5·3. $C_{22}H_{18}O_4$ requires C, 76·2; H, 5·2%).

SUMMARY

1. The occurrence of 3:4-dihydroxyphenylacetic acid in six insect species is reported. Two of these species contain, in addition, 3:4-dihydroxybenzoic acid. These phenolic acids are believed to play a part in the hardening of insect cuticle.

2. From aqueous sulphurous acid used to kill and store two species of *Tribolium*, ethylhydroquinone has been isolated in substantial yield. Its isolation confirms the results of earlier workers who showed that ethyl *p*-benzoquinone is secreted by these insects. Evidence is presented that toluhydroquinone is also present in the extracts.

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The Effect of High Blood Urea on the Acid-base Balance of Serum of Dogs

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In studies on the changes of electrolyte excretion in unilateral tartrate nephrosis and on the added effect of removal of the normal kidney on the acid-base balance, it was thought advisable to determine the effect of high blood-urea values on the acid-base balance of the blood, because possibly the changes in osmotic equilibrium brought about by the increase in urea might change the electrolyte balance. Streicher (1928) found that injections of 10–20% urea solutions in sufficient amounts to raise the blood urea nitrogen of dogs to as high as 328 mg. urea N/100 ml. produced drops in the CO_2 content of the plasma of from 15·3 to 20·8 ml./100 ml. of plasma, the CO_2 estimations being made when the blood urea was from 14 to 130 mg./100 ml. above the normal level. He also reported that the blood chloride and calcium were raised and the potassium lowered.

EXPERIMENTAL PROCEDURE

Dogs weighing from 12 to 19 kg. were kept in metabolism cages during the period of the experiment and were fed their usual mixed diet. Water was given *ad lib.*, the amount taken being measured, and the total fluid output collected and measured. The urea, non-protein nitrogen, total base, CO_2, Cl, inorganic phosphate and protein of serum were determined, immediately before and at varying intervals after the administration of the urea solutions. The blood was obtained from the femoral vein without stasis, withdrawn without contact with air, placed under oil in tubes with constricted necks and centrifuged.

The urea was administered intravenously in 33·3% aqueous solution at 38° and at the rate of 2 ml./min. as described by Leiter (1921). In most dogs the first blood sample was drawn at 10 a.m., the urea given at 10.20 a.m. and subsequent blood samples drawn for analysis at 11.20 a.m. and 4.45 p.m. of the same day. A few experiments were carried on for 3 days. In these the urea was administered at 9.20 a.m. and 4.30 p.m. on the first day, 4.30 p.m. on the second day and at 1.45 p.m. on the third day. Blood samples were drawn at 9 a.m. and 2 p.m. on the first day, at 9 a.m. on the second day and at 9 a.m. (for blood urea estimation only) and at 4.30 p.m. on the third day.

Carbon dioxide was determined by the manometric method of Van Slyke & Neill (1924); chlorides in tungstic acid filtrates by the titrimetric method of Sendroy (1937); and phosphates by the method of King (1932). The serum protein was calculated by subtracting the figure for non-protein nitrogen, estimated by the method of Folin & Wu (1919), from the total nitrogen determined by the usual Kjeldahl procedure using 0·5 ml. of serum. The usual factor of 6·25 was used to convert the figure for protein nitrogen to protein. The method of Van Slyke & Cullen (1914) was used for urea. The total base estimations were done by the method of Stadie & Ross (1925). In the last four experiments determinations were also done by electrodialysis (Consolazio & Talbott, 1940). All determinations were done in triplicate.

Calculations. The CO_2 values were converted from ml./100 ml. serum to mmol./l. by the formula

$$B = CO_2 - \frac{14{\cdot}04\,CO_2}{\text{antilog}\,(pH - 6{\cdot}1)} \times 0{\cdot}713,$$
$$2{\cdot}24$$

as given by Peters, Bulger, Eisenman & Lee (1926). The phosphate in mg. P/100 ml. serum was converted to mmol./l. by the factor 18/31·04 as given by the above authors. The base-combining properties of the protein as m-equiv. were calculated from the formulae of Van Slyke, Hastings, Hiller & Sendroy (1928): $B.P. = 1{\cdot}072 \times g.\ P\ (pH - 5{\cdot}04)$ $(A./G. = 1{\cdot}8)$. In four experiments the pH was determined with the Beckman enclosed glass electrode; in the remainder, a pH of 7·35 was assumed. Although the pH rose during these experiments the maximum error involved by assuming pH 7·35 would be not more than 2 m-equiv., the error in all instances tending to a lowering of the acid column.

RESULTS

Twelve dogs were used. All the dogs remained moderately well during the course of the experiments. There was a marked diuresis in all of them with occasional vomiting and slight diarrhoea in three animals; the results from these three were discarded. The total fluid output of all dogs was slightly less than the total intake. There was no blood in the faeces, and no convulsions occurred. The serum urea rose to levels of 320–590 mg./100 ml. serum 1 hr. from the administration of the urea solution and fell to levels of 160–360 mg. by 4.45 p.m. on the same day. In the 3-day experiments an occasional drop to near the normal level was noted by the morning of the third day.

The results of four typical experiments are shown in Table 1. There was a rise in the pH of the serum varying from 0·03 to 0·08. The effect on the total base and on the total determined acid, as well as the individual constituents of the acid column, was

Table 1. *The changes in acid-base balance accompanying high blood-urea levels produced by urea injections*

Example no.	Wt. of dog (kg.)	Time	pH	Phosphate (mmol./l.)	Chloride (mmol./l.)	Protein (mmol./l.)	CO_2 (mmol./l.)	Total acid (mmol./l.)	Total base (mmol./l.)	Urea (mg./100 ml.)	Urea admin. (g.)
1	19	10.00 a.m.	—	3·1	102·8	16·3	23·9	146·1	152·7	15·0	—
		10.20 a.m.	—	—	—	—	—	—	—	—	95
		11.20 a.m.	—	3·8	102·8	21·8	27·5	155·9	152·2	—	—
		4.45 p.m.	—	3·4	102·8	17·5	25·9	149·6	151·0	—	—
2	17	10.00 a.m.	—	3·7	100·8	16·6	23·9	145·0	147·4	—	—
		10.20 a.m.	—	—	—	—	—	—	—	—	68
		11.20 a.m.	—	4·3	102·5	17·5	25·6	149·9	146·3	540	—
		4.45 p.m.	—	4·6	107·0	18·3	23·7	153·6	157·6	332	—
3	16	10.00 a.m.	7·36	3·5	100·6	14·9	24·5	143·5	153·5 154·1*	19	—
		10.20 a.m.	—	—	—	—	—	—	—	—	64
		11.20 a.m.	7·43	4·2	101·1	15·8	26·2	147·3	150·0 158·2*	486	—
		4.45 p.m.	7·40	4·2	104·2	18·8	25·5	152·7	149·0 161·9*	368	—
4	12	9.00 a.m.	7·35	2·6	108·0	16·1	22·6	149·3	156·6 155·9*	40	—
		9.20 a.m.	—	—	—	—	—	—	—	—	48
		2.00 p.m.	7·41	3·6	108·6	16·1	23·3	151·6	155·6 162·7*	321	—
		4.30 p.m.	—	—	—	—	—	—	—	—	24
		9.00 a.m.	7·39	2·3	109·7	12·9	22·7	147·6	148·9 160·1*	326	—
		4.30 p.m.	—	—	—	—	—	—	—	—	48
		9.00 a.m.	—	—	—	—	—	—	—	58	—
		1.45 p.m.	—	—	—	—	—	—	—	—	48
		4.30 p.m.	7·42	2·6	104·1	14·6	23·4	144·7	140·6 151·9*	311	—

* Total base determined by electrodialysis (Consolazio & Talbott, 1940).

somewhat variable, but some pronounced general tendencies were noted. In the short experiments there was an invariable absolute increase in the total determined acids which was maintained throughout the experimental period in all but one instance. Even in this case (Example 1), however, the determined acids were slightly higher at the end than at the beginning of the experiment. In the first hour there was a definite rise in the phosphate, protein and CO_2 and in all but one experiment (Example 1) the chloride. Between the taking of the second blood sample, 1 hr. after the injection of the urea solution, and the taking of the last specimen, 6¼ hr. afterwards, the CO_2 values usually fell slightly but, as a rule, they did not return to the original level in this time. The protein values usually rose still further in this period, but occasionally a small drop was noted. In all but one instance (Example 1) the chlorides increased more than enough to offset any decreases of CO_2 or protein.

There was a significant effect on the total base. When determined after incineration the total base was always lowered either absolutely or in relation to the sum of the determined acids, the value for the latter often exceeding the total inorganic base. When the total base was determined by electrodialysis, however, it was found that the usual excess of total base over the sum of the determined acids existed (Examples 3 and 4).

DISCUSSION

The difference between the base, as determined after incineration, and the figure obtained by electrodialysis could be accounted for only by the presence of some organic base in the blood. The nature of the extra base has not been determined. That it cannot be ammonia seems certain because the differences observed were too great to be accounted for by any increase in blood ammonia which could be tolerated by the animals, and in one animal in which the blood ammonia was determined no increase was observed. As the urea solution was neutral in reaction and gave no base on electrodialysis the organic base must have been either produced in the animal or have diffused from the tissues into the blood. The observed changes in inorganic base and in the serum anions may be best explained in relation to the presence of this extra base in the blood. Occasional observations of excess of acid over base in the serum have been reported (Peters, Wakeman, Eisenman & Lee, 1929; Atchley & Benedict, 1930; Sunderman, 1931; Hald, 1933). The instances recorded by the first three

groups of authors (Peters *et al.*, Atchley & Benedict, Sunderman) all occurred in individuals with low total base in a variety of conditions—nephritis, diabetic acidosis, pneumonia—while Hald reported the occurrence of an apparent acid excess in some normal individuals. Hald could find no reasonable explanation for the seeming anomaly but as our findings offer reasonable presumptive evidence of the occurrence of an organic base in the blood under one set of experimental conditions, it may be assumed that such base could occur in other conditions. The nature of the base and the mechanisms responsible for its appearance in the blood remain to be determined.

SUMMARY

1. The effect of increases in blood urea produced by the intravenous injection of urea solutions on the electrolyte pattern of the serum was studied.

2. There was an increase in the amounts of the determined acids and a decrease in the amount of total base determined after incineration.

3. When the total base was also determined by electrodialysis no such decrease was found.

4. The difference between the figures for total base obtained by the two methods is taken as reasonable presumptive evidence for the presence of some organic base in the animals with a high blood urea.

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